REMARKS

Reconsideration of this application, is respectfully requested. Claims 1-22 are pending.

Objections to the Specification

The specification was objected to due to typographical issues on pages 20 and 30. Applicants are unsure of the problem and ask the Examiner to please clarify this objection. The printed publication (US 2004/0223949A1) of this application appears to be in order.

Rejection Under 35 U.S.C. 112, First Paragraph

Claims 1 and 3-22 stand rejected under 35 U.S.C. 112, first paragraph for failing to "reasonably provide enablement for treating any cancer wherein a polynucleotide encoding a tumor antigen is administered by any route followed by administration of any cytokine." Within the rejection, the Examiner alleged that an undue burden would be placed upon the skilled artisan in practicing the claimed invention "by any route other than directly to the tumor followed by cytokine." Applicants respectfully disagree and traverse this rejection as indicated below.

Applicants respectfully disagree with the Examiner as to any lack of enablement and maintain that the claims are enabled. Part (a) of claim 1 requires administration of a nucleic acid encoding a tumor antigen such that the host develops an immune response thereto. Selection of the tumor antigen is a key determinant for each particular type of cancer; the process of selecting a tumor antigen would not place an undue burden upon the skilled artisan. In fact, the specification provides list of exemplary tumor antigens (i.e., paragraph 0018 of US 2004/0223949A1). As described therein, each of these tumor antigens is known by those of skill in the art to be associated with at least one particular type of cancer. For instance, it is known that gp100 and the MAGE genes are expressed in melanoma, CEA is expressed in certain colorectal tumors, and PSA is expressed in prostate cancers. It is certainly within the skill set of the highly-trained scientists involved in this field to select a tumor antigen, and therefore a type of cancer, in which to

apply the claimed method. Accordingly, Applicants believe the claimed method is enabled with respect to the application thereof to "any cancer".

With respect to the use of "any route", the Examiner alleged on p. 5 of the Office Action that "[t]he choice of a particular route of administration suitable for treatment of one type of cancer may not be applicable to the treatments of other cancers." The claimed invention is not dependent upon a particular route of administration (i.e., direct administration to a particular site). It is the combination of the administration of a nucleic acid encoding a tumor antigen and the subsequent administration of a high dose of cytokine that is inventive. All that is required in part (a) of claim 1 is that a nucleic acid encoding a tumor antigen is administered such that the host develops an immune response to the tumor antigen. As stated in the specification, many suitable routes of administration are in fact available to one of skill in the art. Even if it were true that a particular route of administration suitable for one type of cancer was not suitable to every other type of cancer, it would not be an undue burden for the skilled artisan to select another route suitable to that other type of cancer. As suggested in Applicants' specification and the articles cited by the Examiner (see below), many such routes are known in the art.

The instantly claimed invention does not require expression at any particular site in the patient. In contrast to the Examiner's allegations, the administration of nucleic acids to induce an immune response has been successful. For instance, Pouton et al. (Adv. Drug Del. News, 46 (2001) 187-203; cited by the Examiner), make multiple statements supporting the feasibility of immune-base nucleic acid delivery systems, such as:

> ...small viral vectors are able to transfect some tumors on a widespread basis after a single injection. (p. 192, col. 2, second paragraph)
>
> Subcutaneous administration of membrane-impermeable agents is a useful means of delivering materials into the lymphatic system. . . . Hence, it seems likely that subcutaneous administration of vectors for gene delivery would lead to substantial retention of the material at or near the site of injection, and the possibility should therefore be approached with caution. This phenomenon may be

used to advantage for local effect, such as for peritumoral expression of immune modulators. (p. 192, col. 2, third paragraph to page 193, first column, first paragraph)

Phagocytosis is the likely fate of any lipoplexes (or viral particles) that reach the alveoli. Should there be a clinical indication for gene delivery to alveolar macrophages, phagocytosis may be regarded as a passive process that could be exploited. (p. 193, col. 2, first paragraph)

Particle-mediate delivery was introduced in the late 1980s [118] and has been developed fro in vivo transfection experiments in mammalian species [119,120]. . . . This approach is particularly promising for genetic vaccine development using the direct introduction of DNA to antigen-presenting cells, such as the Langerhans cells of the skin [121, 122]. (p. 198, col. 1, third paragraph)

Immunotherapeutic approaches are attractive because they do not depend on transfection of all tumour cells. (p. 198, second column, third paragraph)

Similarly, Crystal (Science, Vol. 270, pp. 404-410 (1995); cited by the Examiner) touts the feasibility of gene transfer in immunological applications, stating:

Once considered a fantasy that would not become a reality for generations, human gene transfer has moved from feasibility and safety studies in animals to clinical applications more rapidly than expected by even its most ardent supporters (1-3). (p. 404, col. 1 second paragraph)

Probably the most remarkable conclusion drawn from the human trials is that human gene transfer is indeed feasible. (p. 405, col. 3, paragraph 2)

. . . several studies have demonstrated that therapeutic genes transferred to humans by means of retrovirus, adenovirus, and plasmid-liposome vectors can evoke biologic response that are relevant to the gene product and to the specific disease state of the recipient (Table 2).

There are also studies in which human gene transfer appears to have initiated biologic responses that are relevant to therapy for an acquired disorder. These are all "tumor vaccine" studies, based on

> the hypothesis that exaggerated local expression of an immune related cytokine might help activate the immune system sufficiently to recognize tumor antigens and control the growth of tumor cells. (p. 408, col. 1, paragraph 2)
>
> Clinical experience to date suggests that retrovirus, adenovirus, and plasmid-liposome vectors all need refinement, but each is relatively well suited for the clinical targets at which they have been directed. (p. 409, col. 2, paragraph 4)

In addition, the successful administration of tumor antigens to human beings by several different routes (i.e., intradermal, subcutaneous, intranodal, and intravenous) has been shown by, for example, Marshall, et al. (J. Clin. Oncol. 18(23): 3964-3973 (2000)), van der Burg, et al. (Clin. Cancer Res., 8: 1019-1027 (2002)), Astatsturov, et al. (the Applicants; Clin. Cancer Res. 9: 4347-4355 (2003)), Karakinas, et al. (J. Immunol. 171: 4898-4904 (2003)), van Baren, et al. (J. Clin. Oncol. 23 (35): 9008-9021 (2005)). Thus, the skilled artisan would have many routes to choose from in practicing the claimed invention.

The Examiner further alleges that the instant claims are not enabled as to "any cytokine". Applicants respectfully disagree. The specification provides a list of potentially useful cytokines at paragraphs 0044 and 0045 (US 2004/0223949A1). Many others are known in the art. The suitability of any particular cytokine to any particular type of cancer may involve some experimentation, but Applicants do not believe such experimentation would be undue.

Applicants were the first to coordinate the use of a nucleic acid encoding a tumor antigen and high doses of cytokine(s) to produce an anti-tumor immune response. With Applicants' description of the method in hand, the burden placed upon the skilled artisan in identifying and selecting particular components to use therein would not be undue. Accordingly, Applicants believe the claimed invention is enabled and respectfully request that this rejection be withdrawn.

Rejection Under 35 U.S.C. 112, Second Paragraph

Claims 1 and 3-22 stand rejected under 35 U.S.C. 112, second paragraph with respect to the term "high dose of a cytokine". The Examiner alleged that the meaning of

the term "high dose of a cytokine" is unclear in claim 1 and the specification. Applicants respectfully disagree and traverse this rejection as indicated below.

As indicated by the specification, the term "high dose" is not tied to any particular amount of cytokine. The meaning of the term "high dose", in its most basic form, is a dose above what is commonly known to be a low dose. While the exact amount of cytokine falling within the meaning of "high dose" may not be entirely consistent within the art, ranges corresponding thereto are art-recognized for several cytokines. It is understood that such ranges vary depending on the particular cytokine. The specification describes the parameters of treatment with high dose interferon (paragraph 0072 of US 24223949A1; Kirkwood, et al. J. Clin. Oncol. 14: 7-17 (1996) and J. Clin. Oncol., 18(12): 2444-2458 (2000)). The meaning of a "high dose" of IL-2 is art-recognized (see, for example, Lindsey, et al. J. Clin. Oncol. 18(9): 1954-1959 (2000); Phan, et al. J. Clin. Oncol. 19(15): 3477-3482 (2001); McDermott, et al. J. Clin. Oncol. 23(1): 133-141 (2005)). Similar information for determining the nature of a high dose is available for other cytokines including, for example, GM-CSF (Abramovich, et al. Abstract No. 205 of 1999 ASCO Meeting), IL-11 (Kurzrock et al. J. Clin. Oncol. 19(21): 4165-4172 (2001)), and TNF-alpha (Rossi, et al. Ann. Surg. Oncol. 11(2): 173-177 (2004)). Applicants do not believe this phrase would be unclear to one of skill in the art. As such, withdrawal of this rejection is respectfully requested.

Rejection Under 35 U.S.C. 102(b)

Claims 1, 3-8 and 14-15 stand rejected under 35 U.S.C. 102(b) as being anticipated by Paoletti (U.S. Pat. No. 5,942,235). Applicants respectfully disagree and traverse this rejection as indicated below.

Paoletti relates generally to the use of viral vectors to express cytokines and tumor-associated antigens in mammalian cells. With regard to claims 1 and 3, the Examiner points to Paoletti at col. 16, lines 3-8 as teaching administration of a cytokine from a modified tumor cell. At that section, Paoletti states:

> Tumor cells can be modified to express TAAs, cytokines, or other novel antigens (i.e. class I or class II major histocompatibility genes). Such modified tumor cells can subsequently be utilized for active immunization. The

> therapeutic potential for such an administration is based on the ability of these modified tumor cells to secrete cytokines and to alter the presentation of TAAs to achieve systemic anti-tumor activity.

Instant claim 1, upon which claim 3 depends, requires the administration of a composition for inducing an immune response against a tumor antigen, and "subsequently administering to the host a high dose of a cytokine". The Examiner has not shown that Paoletti teaches the claimed method. At most, Paoletti only teaches the simultaneous administration of antigen and cytokine. The subject matter of claim 1 is distinct from the teaching of Paoletti. As such, it is respectfully requested that this rejection be withdrawn.

As to claims 4-8 and 14-15, the Examiner alleges that Paoletti teaches poxvirus vectors expressing TAAs with or without specific cytokines. The viral vector encodes both TAAs and cytokines which are simultaneously administered (as both are contained in the same vector). Claims 4-8 and 14-15 are dependent upon claim 1, which requires initially administering a nucleic acid encoding a tumor antigen and "subsequently administering to the host a high dose of a cytokine". The Examiner has not shown that Paoletti teaches all steps of the claimed method. The subject matter of claims 4-8 and 14-15 is distinct from the teaching of Paoletti. As such, it is respectfully requested that this rejection be withdrawn.

Rejections Under 35 U.S.C. 103(a)

A. Paoletti (U.S. Pat. No. 5,942,235) and Kirwood (J. Clin. Oncol. 19(9): 2370-80, 2001)

Claims 1, 9-10 and 16-22 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Paoletti (US 5,942,235) in view of Kirkwood (J. Clin. Oncol. 19(9): 2370-80 (2001)). Applicants respectfully disagree and traverse this rejection as indicated below.

The Examiner alleges that Paoletti teaches attenuated viruses for expressing tumor antigens and that cytokine secreted from the tumor cells may be used for active immunization. The Examiner also alleges that Kirkwood teaches the use of high-dose

interferon α2b in the treatment of melanoma. Thus, the Examiner alleges, one would have been motivated to combine these teachings to make the claimed invention, and would have had a reasonable expectation of success in doing so. Applicants respectfully disagree.

The Examiner acknowledged that: 1) Paoletti does not teach administration of high dose interferon α2b; and, 2) Kirkwood does not teach combining high dose interferon α2b cytokine therapy with expression of a tumor antigen to treat cancer. Applicants agree. In addition, and in contrast to the Examiner's allegations, Applicants do not believe the skilled artisan would have had a reasonable expectation of success in practicing the claimed invention until the method was actually carried out and the results thereof observed. There was no reason to believe that administration of high-dose cytokine following the initial immune response resulting from administration of the nucleic acid encoding the tumor antigen would result in the clinical results observed by the Applicants. While one may have been motivated to <u>try</u> combining the work of Paoletti and Kirkwood, there could not have been a reasonable expectation that the method would function as desired. It was not until the Applicants actually did so that there was any reasonable expectation of success in practicing the claimed method. As this expectation was not present until Applicants carried out the experiments, the claimed method cannot be obvious by Paoletti and Kirkwood. As such, it is respectfully requested that this rejection be withdrawn.

B. Paoletti (U.S. Pat. No. 5,942,235) and Schlom (U.S. Pat. No. 6,045,802)

Claims 1, 3-7 and 11-13 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Paoletti (U.S. Pat. No. 5,942,235) in view of Schlom (U.S. Pat. No. 6,045,802). Applicants respectfully disagree and traverse this rejection as indicated below.

The Examiner alleges that Paoletti teaches attenuated viruses for expressing tumor antigens and that cytokine secreted from tumor cells may be used for active immunization. The Examiner also alleges that Schlom teaches the use of gp100 in recombinant viral vectors. Thus, the Examiner alleges, one would have been motivated

to combine these teachings to make the claimed invention, and would have had a reasonable expectation of success in doing so. Applicants respectfully disagree.

The Examiner acknowledged that: 1) Paoletti does not teach expression of gp100 from a viral vector; and, 2) Schlom does not teach administration of a cytokine subsequent to administration of a tumor antigen. Applicants agree. The Examiner has not set forth where in Paoletti or Schlom the second step in the claimed method is taught. The Examiner has merely stated that Paoletti teaches the use of viral vectors to express tumor antigens and that Schlom teaches expression of gp100. However, the Examiner has not described where Paoletti and / or Schlom teach the use of high dose cytokine subsequent to administration of the nucleic acid encoding a tumor antigen. Accordingly, Applicants respectfully maintain that this rejection is improper and request its withdrawal.

CONCLUSIONS

Reconsideration of this application is respectfully requested. Applicants believe the claims are in condition for allowance and respectfully request the issuance of a Notice of Allowance as soon as possible. The Examiner is encouraged to contact the undersigned if it is believe doing so would expedite prosecution of this application.

Respectfully submitted,

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Phase I Study in Advanced Cancer Patients of a Diversified Prime-and-Boost Vaccination Protocol Using Recombinant Vaccinia Virus and Recombinant Nonreplicating Avipox Virus to Elicit Anti-Carcinoembryonic Antigen Immune Responses

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Purpose: **This trial sought to determine, for the first time, the validity in human vaccinations of using two different recombinant vaccines in diversified prime-and-boost regimens to enhance T-cell responses to a tumor antigen.**

Patients and Methods: **Eighteen patients with advanced tumors expressing carcinoembryonic antigen (CEA) were randomized to receive either recombinant vaccinia (rV)-CEA followed by three avipox-CEA vaccinations, or avipox-CEA (three times) followed by one rV-CEA vaccination. Subsequent vaccinations in both cohorts were with avipox-CEA. Immunologic monitoring was performed using a CEA peptide and the enzyme-linked immunospot assay for interferon gamma production.**

Results: **rV-CEA followed by avipox-CEA was superior to the reverse order in the generation of CEA-specific T-cell responses. Further increases in CEA-specific T-cell precursors were seen when local granulocyte-macrophage colony-stimulating factor (GM-CSF) and low-dose interleukin (IL)-2 were given with subsequent vaccinations. The treatment was extremely well tolerated. Limited clinical activity was seen using vaccines alone in this patient population. Antibody production against CEA was also observed in some of the treated patients.**

Conclusion: **rV-CEA was more effective in its role as a primer of the immune system; avipox-CEA could be given up to eight times with continued increases in CEA T-cell precursors. Future trials should use rV-CEA first followed by avipox-CEA. Vaccines specific to CEA are able to generate CEA-specific T-cell responses in patients without significant toxicity. T-cell responses using vaccines alone may be inadequate to generate significant anticancer objective responses in patients with advanced disease. Cytokines such as GM-CSF and IL-2 may play a key role in generating such responses.**

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CARCINOEMBRYONIC antigen (CEA) is a 180,000 molecular weight oncofetal glycoprotein expressed in the normal fetal colon. In adults, CEA has been found in lower levels in normal colonic mucosa and also in saliva, feces, serum, and colonic lavages.[1] CEA is overexpressed in virtually all colorectal adenocarcinomas and most adenocarcinomas of the pancreas, stomach, breast, and lung.[2-4] Many colorectal cancers and some carcinomas at other sites produce high levels of CEA that are measurable in sera.[5] Because of this, CEA is one of the most widely used serologic markers of malignancy, especially in patients with colorectal cancer.

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It has been proposed that CEA functions as an intercellular recognition and adhesion molecule.[6] Increased CEA expression by a group of cells may promote metastasis through increased intercellular adhesions mediated by CEA. After metastasizing from a primary tumor, a group of adhesive cells may more easily survive to reach a distant organ and form a secondary tumor.

Using CEA as a target in immunologic-based therapies has two potential problems. First, given that CEA is a normal protein expressed in the body, it is likely that tolerance will exist to this protein. Secondly, if one were successful in generating such an immune response, the result could lead to autoimmune disease. On the other hand, if one were successful at this, the impact of such therapy would have tremendous clinical implications. Thus immunotherapy protocols are being designed to produce an immune response against CEA-bearing cancer cells by generating cytotoxic T lymphocytes (CTL) that lyse CEA-expressing cancer cells while sparing the normal CEA-expressing gut cells. This may be possible because CEA is expressed at higher levels in carcinoma cells versus normal colonic epithelial cells.[7] A recombinant vaccinia virus containing the CEA gene (designated rV-CEA) has been

developed.[8,9] This virus is capable of infecting professional antigen-presenting cells (APCs) and presenting CEA peptides to T lymphocytes in the context of HLA class I and II molecules, which in turn activate the corresponding $CD8^+$ or $CD4^+$ T cells.[8,10,11] The safety of rV-CEA has been documented in nonhuman primates.[11] In a phase I clinical trial, the safety of rV-CEA was demonstrated in humans; however, no significant antineoplastic effect was observed.[11-13] Possible reasons for the lack of clinical efficacy in these trials were (1) prior exposure to the vaccinia virus in all patients treated, which led to the development of antivaccinia immune responses on repeated dosings of the vaccine, (2) the advanced state of the tumors in patients, and (3) potentially compromised immune status of patients owing to prior chemotherapy regimens.

The phase I rV-CEA study demonstrated that CEA-specific T-cell responses could be generated in humans through administration of a vaccine.[11] This study also showed that CTL cell lines could be generated from peripheral-blood mononuclear cells (PBMCs) of rV-CEA–vaccinated patients in the presence of a CEA peptide, designated carcinoembryonic antigen peptide-1 (CAP-1). This 9-mer amino acid peptide (YLSGANLNL) has been shown to bind HLA-A2 class I molecules. Tumor cells expressing HLA-A2 molecules and CEA were lysed by CAP-1–specific CTL from HLA-A2–positive vaccinated patients, whereas non–HLA-A2–expressing cells were not lysed. This finding indicated that CTL-mediated lysis occurred in a major histocompatibility complex–restricted manner. Stable CTL lines derived by culture of PBMCs from rV-CEA–vaccinated patients with CAP-1 peptide and interleukin (IL)-2 have also been described.[14,15] Recently, a CAP-1 agonist epitope has been identified and designated (CAP-1-6D), which has been shown to activate T cells to even higher levels.[16,17]

Another recombinant anti-CEA vaccine, avipox-CEA, has been developed.[9,18] The canarypox vector used in this trial has been termed ALVAC. Similar to rV-CEA, avipox-CEA contains the CEA gene in its genome but, unlike rV-CEA, cannot replicate in mammalian cells. Avipox viruses, such as ALVAC and fowlpox, infect mammalian cells, express their transgene product for 14 to 21 days before death of the cell, and then do not infect other cells. Therefore, systemic infections and the resulting influenza-like symptoms as seen with rV-CEA do not occur. Additionally, humans are unlikely to have had prior exposure to this virus. The safety of avipox-CEA has been documented in a phase I trial in patients with advanced carcinomas.[19] A moderate but statistically significant increase in the number of CEA-specific CTL precursors was observed in seven of nine HLA-A2–positive patients treated with avipox-CEA; however, no true, objective anticancer effects were seen. Possible explanations for the low number of CTL precursors observed include decreased immune status and/or preexisting immune suppression related to the advanced state of disease in the patients studied.

Preclinical evidence has indicated that the combination of rV-CEA and avipox-CEA in diversified prime-and-boost protocols would in fact generate a more vigorous T-cell response than either vaccine alone.[18] When rV-CEA was used to prime the immune system and avipox-CEA was used as a boost in the experimental model, CEA-specific T-cell responses were at least four times greater than those achieved with three vaccinations of avipox-CEA alone. Multiple boosts of avipox-CEA further potentiated these CEA-specific T-cell responses.[18] This preclinical finding, combined with the results of the phase I trials using either rV-CEA or avipox-CEA alone, justified a phase I trial to validate this concept of diversified prime-and-boost vaccination protocol for the first time in patients with advanced carcinomas. Preclinical data also demonstrated that granulocyte-macrophage colony-stimulating factor (GM-CSF) and low-dose IL-2 can potentiate the CEA-specific immune responses to rV-CEA vaccinations; little, if any, effect was seen when the cytokines were used alone.[20,21]

In this study, we proposed to treat cancer patients with CEA-bearing tumors with rV-CEA (V) and avipox-CEA (A) to determine (1) the safety of the two agents in this population, (2) whether the sequence of administration (ie, VAAA *v* AAAV) has an effect on T-cell response, and (3) whether any objective responses could be achieved using vaccines alone in patients with metastatic disease. Although preclinical evidence supports the addition of cytokines to these vaccines,[20,21] our initial studies were performed with the vaccines alone to first document safety of the diversified prime-and-boost vaccine combination. An enzyme-linked immunospot (ELISPOT) assay was selected to monitor CEA-specific T-cell responses to a CEA 9-mer peptide. The ELISPOT assay used for interferon gamma (IFN-γ) production required only a 24-hour in vitro stimulation of PBMCs from patients, either pre- or postvaccination. An identical ELISPOT assay to an influenza (Flu) 9-mer peptide was used simultaneously as a control.

PATIENTS AND METHODS

Patient Eligibility

To be eligible for this trial, patients had to meet the following criteria: pathologic evidence for advanced, incurable (or high-risk) malignancy (patients with stage IV malignancy but without radiographic evidence of disease were eligible); serum CEA at least 10 ng/mL at some point in the past or tumor that stained positively for CEA by immunohistochemical techniques; age at least 18 years;

anticipated survival of at least 6 months; ability to give informed consent; performance status of 0 or 1 (Eastern Cooperative Oncology Group); WBC count of at least 3,000/μL and platelet count of at least 100,000/μL; prothrombin time and partial thromboplastin time within normal ranges; normal serum creatinine level or creatinine clearance at least 60 mL/min; adequate immunologic function, defined by normal delayed-type hypersensitivity, normal CD4:CD8 ratio (> 1) or normal immunoelectrophoresis; human immunodeficiency virus seronegativity; no other diagnoses of altered immune function; no prior radiation to more than 50% of all nodal groups; and no concurrent use of corticosteroids. Contraindications to enrollment included history of another malignancy in the past 2 years, prior radiation to the pelvis, recent major surgery, pregnancy or lactation, serious intercurrent illness, and clinically evident brain metastasis. Patients who received avipox-CEA in a previous clinical trial were able to participate in this trial, provided that they still met the eligibility criteria. Three such patients were enrolled; these patients were enrolled to explore the role of a delay in vaccinations on the immune system T-cell response and were evaluated separately from the other patients in this trial. These patients are clearly identified in the Results section, and results from these patients were not included in the evaluations to define the optimal prime-and-boost protocol.

Treatment

Twelve HLA-A2–positive patients were initially selected for this study because of requirements of the T-cell assay used for monitoring purposes (see Immunologic Monitoring Methods: ELISPOT and Antibody Assays). It was unknown whether priming the immune system with a more potent immunogen (rV-CEA) would produce a greater T-cell response compared with boosting the immune system with this immunogen. Therefore, patients were randomly assigned to one of two study cohorts. The first cohort received one vaccination of rV-CEA followed by three vaccinations of avipox-CEA (designated VAAA). The second cohort received vaccinations in the reverse order (AAAV). Three HLA-A2–negative patients were randomized to each cohort to expand the clinical experience in HLA-A2–negative patients and further document safety. All vaccinations were administered 4 weeks apart, with each 28-day period constituting a cycle. Cycles of 28 days were considered optimal, because longer intervals would be unacceptable for cancer patients and shorter intervals might lessen the immune response. rV-CEA (1.0×10^7 pfu) was administered intradermally into the deltoids. Avipox-CEA (2.5×10^7 plaque-forming units [pfu]) was administered subcutaneously in two equally divided doses (for volume purposes), using the Biojector 2000 (Bioject Inc., Portland, OR) needle-free system, into the arm, thighs, or buttocks (the injection site was rotated). Dose levels for both vaccines were documented in previous clinical trials.[11,19] Patients in both cohorts were monitored before each injection and 4 weeks after the final injection by physical examination, measurement of performance status, complete blood cell counts, blood chemistry profile, urinalysis, and measurement of CEA level; PBMCs for T-cell immunologic monitoring and serum for measuring antibody production against CEA were also taken at these time intervals. Tumor responses were evaluated after every two treatment cycles.

Investigational Use of GM-CSF and IL-2 as Vaccine Adjuvants

Preclinical studies with vaccines have shown that local administration of GM-CSF at the vaccination site[21-23] or systemic administration of low-dose IL-2 after rV-CEA vaccination[20] enhanced CEA-specific CTL responses when compared with the use of rV-CEA alone. After undergoing four cycles of vaccine treatment (VAAA or AAAV), patients who had no evidence of progressive disease could elect to continue to receive vaccinations with avipox-CEA with GM-CSF (Leukine; Immunex Corporation, Seattle, WA) for two cycles. GM-CSF was prepared by reconstituting GM-CSF in lyophilized powder form with sterile water to a concentration of 500 μg/mL for injection per United States Pharmacopeia. GM-CSF 100 μg was patient-administered as close to the most recent vaccination sites (ie, both arms) as possible, beginning on the day of avipox-CEA vaccination and for three consecutive days thereafter. If patients continued to show no further disease progression after two cycles, patients could elect to receive avipox-CEA with GM-CSF and IL-2 (Proleukin; Chiron, Emeryville, CA). IL-2 was prepared by reconstituting lyophilized IL-2 to a final concentration of 18.0×10^6 IU/m^2. The solution was stored at 4°C and administered at room temperature within 48 hours of reconstitution. IL-2 was patient-administered on days 7 through 11 of each cycle at a dosage of 6.0×10^6 IU/m^2. Both the GM-CSF and IL-2 were provided to us by the Cancer Therapy Evaluation Program of the National Cancer Institute, Bethesda, MD.

Vaccine Preparation

rV-CEA is a live vaccinia virus prepared from the Wyeth New York Board of Health strain of vaccine. It has been genetically engineered using a plasmid vector to carry a copy of the human CEA gene in the viral 30K gene (Hind III M fragment). The vaccine was manufactured by Therion Biologics Corporation (Cambridge, MA). Virus for vaccination was grown in the CV-1 monkey kidney cell line. The vaccine was stored in vials of 1.0×10^9 pfu/mL. Vials were kept at −70°C until the day of administration and thawed before use at room temperature, at which point 10 μL (1.0×10^7 pfu) of the vaccine was administered intradermally into the deltoids. Any remaining units of vaccine were stored at 4°C for no more than 4 days. Vaccine preparation was performed in a sterile hood.

Avipox-CEA is a recombinant canarypox virus (ALVAC) that contains the entire human CEA gene. The vaccine was manufactured by Pasteur-Mérieux Serums et Vaccins (Marcy, France)/Virogenetics (Troy, NY). The canarypox strain from which ALVAC was derived was first isolated at the Rentschler Bakteriologisches Institute (Laupheim, Württemberg, Germany), where it was attenuated by serial passage in chick embryo fibroblasts. The recombinant virus was grown and generated on chick embryo fibroblasts from pathogen-free flocks qualified for vaccine production. The vaccine was stored in vials of 2.5×10^7 pfu/0.2 mL. Vaccine vials were kept at −70°C until the day of administration. They were then thawed at room temperature or in a 37°C water bath. The sample in the vial was diluted with sterile saline to a total volume of 500 μL and then divided into two 250-μL syringes for the Bioject system. Dilutions were performed in a sterile hood.

Immunologic Monitoring Methods: ELISPOT and Antibody Assays

Normal HLA-A2 donor PBMCs were obtained from the Clinical Center blood bank of the National Institutes of Health. Normal and patient PBMC samples were stored in liquid nitrogen at a concentration of 1×10^7 cells/mL. Cells were thawed and cultured overnight in RPMI-1640 complete medium (Life Technologies, Inc, Gaithersburg, MD) at 37°C at 5% CO_2 before performing the ELISPOT assay. A modification of the ELISPOT assay, measuring IFN-γ production, was used to determine the T-cell CTL precursor frequency specific for the CAP-1-6D peptide[16,17] in both pre- and postvaccination PBMCs. Briefly, 96-well millimeter high-affinity plates (Millipore Corporation, Bedford, MA) were coated with 100 μL/well of capture monoclonal

antibody against IFN-γ at a concentration of 10 μg/mL for 12 hours at room temperature. Plates were blocked for 30 minutes with RPMI 1640 plus 10% human antibody serum. A total of 2×10^5 PBMCs were added to each well. CAP-1-6D–pulsed C1*R*-A2 cells were added into each well as APC at an effector:APC ratio of 1:3. Unpulsed C1R-A2 cells were used as a negative control. HLA-A2 binding Flu matrix peptide 59-66 was used as a positive peptide control.[24] Cells were incubated for 24 hours and lysed with phosphate buffered saline (PBS)-Tween (0.05%). Biotinylated anti–IFN-γ diluted to 2 μg/mL in PBS-Tween containing 1% bovine serum albumin (BSA) was added and incubated overnight in 5% CO_2 at 37°C. Plates were washed three times and developed with avidin alkaline phosphatase (GIBCO/BRL, Grand Island, NY) for 2 hours. After washing the plates three times, each well was examined for positive dots. The number of dots in each well was counted by two separate investigators in a blinded manner, and the frequency of responding cells was determined for a total of 1.2×10^6 effector cells plated.

Western Blot Analysis

Purified preparations of native CEA, recombinant CEA, and BSA (1 μg of each) were electrophoresed on denaturing sodium dodecyl sulfate/4% to 20% gradient Trisglycine polyacrylamide gels (Novex, San Diego, CA) and electroblotted using Trisglycine transfer buffer (Novex) to nitrocellulose membranes (0.45-μm pore size; Novex) for 2 hours at 4°C. A See-Blue stain marker (Novex) was included as a molecular weight standard on all membranes. The membranes were incubated overnight at 4°C in PBS containing 5% BSA to prevent nonspecific protein binding. This and each additional incubation and washing were performed on a shaking apparatus. The membranes were then washed four times (10 min/wash) with PBS containing 0.025% Tween-20 (Bio Rad Laboratories, Hercules, CA). Patient serum was diluted in PBS containing 1% BSA and 5% normal goat serum (Life Technologies) and incubated with the membrane for 5 hours at room temperature. Pooled normal human sera (Gemini Bio Products, Calabasas, CA), the HuCol-1 anti-CEA antibody (1 μg/mL), and human immunoglobulin G (IgG; 1 μg/mL) (Jackson ImmunoResearch, West Grove, PA) were used as controls. Each membrane received 15 mL of appropriately diluted patient sera or controls. Membranes were then washed sequentially under stringent conditions (10 min/wash) with PBS containing 0.3%, 0.1%, 0.05%, and 0.025% Tween-20. The wash with 0.025% Tween-20 was performed twice. Goat antihuman IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was diluted 1:20,000 in PBS containing 1% BSA and was incubated with the membranes for 1 hour at room temperature. The membranes were washed four times (10 min/wash) at room temperature and then kept overnight at 4°C with PBS containing 0.025% Tween-20. The membranes were first treated with the electrochemiluminescence detection reagent (Amersham Life Sciences, Arlington Heights, IL) and then exposed to Kodak Biomax MR film (Eastman Kodak, Rochester, NY) and developed.

Statistical Methods

Eighteen patients (12 HLA class I $A2^+$ and six HLA class I $A2^-$) were randomized to either the VAAA or AAAV study cohort. Once accrued to the study, each patient was randomly assigned to either cohort with the stipulation that no more than nine patients could comprise each cohort. A statistical comparison of the two study cohorts (VAAA and AAAV) was performed by assuming that the distribution of ln(change in CEA-specific T-cell precursor frequency) was approximately normal and that it was reasonable to analyze the postvaccination precursor frequency minus the prevaccination frequency for each sample as representing the change in precursor frequency for each patient. For example, the change in CEA-specific T-cell precursor frequency for patient no. 4 would be postvaccination ln(1/87,500) − prevaccination ln(1/200,000). This analysis revealed the percentage increase or decrease in precursor frequency for each patient pre- versus postvaccination. The two study cohorts were compared by calculating the mean of the percentage increase or decrease in precursor frequency for each cohort.

Table 1. Patient Characteristics: ALVAC/Vaccinia

Characteristic		No. of Patients
Age, years		
Range	24-83	
Median	60.5	
Performance status		
0		13
1		5
Sex, male/female		10/8
Prior therapy		
Chemotherapy		18
1 prior regimen		8
2 prior regimen		6
> 2 prior regimen		4
Radiation		8
Primary site		
Colorectal		11
Pancreas		1
Stomach/esophagus		3
Cervix		1
Breast		2
HLA-A2 +/−		12/6
Tumor burden		
Visceral metastases		11
No evidence of disease		7

RESULTS

Eighteen patients were enrolled in this phase I study. Patient characteristics are listed in Tables 1 and 2. Of 18 patients, nine were randomized to receive VAAA (cohort 1). The remaining patients were randomized to receive AAAV (cohort 2). Six of the patients in each cohort were HLA-A2–positive for immunologic monitoring purposes. Three HLA-A2–negative patients were randomized to each cohort to expand the safety profile in HLA-A2–negative patients. Patient no. 17 (AAAV, HLA-A2–positive) was removed from the study after two vaccinations because of disease progression and was not replaced because sufficient toxicity data were obtained from the other patients in the AAAV cohort. Patients no. 3 (VAAA) and 9 (AAAV), both HLA-A2–negative, were also removed from study because of disease progression. All other patients received four monthly doses of the vaccines according to their cohort schedule. Nine patients with no evidence of disease progression at the completion of the initial four cycles of vaccinations elected to continue receiving avipox-CEA with GM-CSF. Seven of these patients elected to add IL-2 to the treatments after two cycles of avipox-CEA with GM-CSF.

Table 2. Clinical Results

Cohort	Patient No.	Tumor Site	Serum/Tumor CEA*	HLA-A2+	ED	DTH+	CD4:CD8 Ratio	Treatment Duration (no. of cycles)†	CEA Level (ng/mL) Pre-/Posttreatment
VAAA	4	Esophagus	T	Y	Y	+	391:454	4	0.7 /0.8
	5	Breast	S	Y	Y	+	363:330	6	75.4 /910
	14	Colon	T	Y	N	+	217:466	6	0.4 /1.1
	15	Cervix	T	Y	Y	+	1346:650	10	7.8 /2.7
	18	Colon	T	Y	N	−	105:32	4	3.2 /8.1
	19	Colon	T	Y	N	+	1590:750	10	3.6 /103.4
	3	Esophagus	S	N	Y	+	199:143	2	2,158 /ND
	6	Colon	S	N	N	+	745:134	21+	0.6 /0.7
	7	Colon	S	N	N	+	882:470	20+	2.5 /54.4
	10‡	Colon	S	Y	N	+	942:471	4	0.5 /3.2
	11‡	Unknown primary	S	Y	Y	+	725:637	8	26 /14.6
	13‡	Stomach	T	Y	N	+	562:323	1	0.4 /ND
AAAV	2	Rectum	T	Y	N	+	725:445	10	2.5 /3.3
	12	Breast	S	Y	Y	+	421:251	4	15,770 /18,944
	16	Colon	S	Y	Y	−	649:176	4	28.7 /185.9
	17	Colon	S	Y	Y	−	475:289	2	936/2,361.2
	20	Colon	S	Y	Y	+	466:513	4	275 /545.5
	21	Pancreas	T	Y	N	+	352:181	7	0.5 /<0.5
	1	Colon	T	N	Y	−	201:158	8	0.5 /4.0
	8	Colon	S	N	Y	+	300:114	4	95.3 /199.3
	9	Colon	S	N	Y	+	493:108	2	575.4 /487.1

Abbreviations: ED, evidence of disease before treatment began; DTH, 10 mm in duration at 48 hours after skin testing with antigen panel; ND, not determined; Y, yes; N, no.

*S = CEA level in serum > 10 at any point in course of disease; T = CEA+ on immunohistochemical testing of tumor samples.

†A plus (+) in the treatment duration column indicates treatment is ongoing during preparation of this manuscript.

‡Patient who received ALVAC-CEA during a previous clinical trial.

Toxicity

Both treatment schedules were well tolerated in all patients studied. No significant toxicity could be attributed to the treatment in either cohort. Commonly, mild skin reactions, which lasted for 3 to 5 days, were noted after rV-CEA and avipox-CEA vaccination. No systemic toxicity was observed when avipox-CEA and GM-CSF were administered together, although increased skin reactions occurred. Typical IL-2 toxicity, consisting of low-grade fevers, chills, fatigue, nausea, and skin reactions, was noted when IL-2 was administered with avipox-CEA and GM-CSF. Four of nine patients elected to discontinue IL-2 on subsequent vaccinations because of unacceptable toxicity related to IL-2 (all toxicity was grade 1 or 2, with the majority being grade 1 fever and fatigue) and continued to receive avipox-CEA with GM-CSF for subsequent cycles. With the exception of those cycles given with IL-2, no significant toxicity was observed in any patients.

Clinical Response

No objective antitumor responses were observed in any patients treated. Two of the patients (nos. 6 and 7) from these two cohorts remain on study currently and are being treated with monthly cycles 16 and 17, respectively. However, because both patients are HLA-A2–negative, we cannot determine their T-cell response. Patient no. 6 has metastatic colon cancer to his liver and was vaccinated after having a complete radiographic response to fluorouracil in October 1997. He remains without evidence of disease with a serum CEA level of 0.7 more than 21 months after his initial vaccination. Patient no. 7 had metastatic colon cancer to his liver, which was resected, and has now been on the vaccine study for more than 20 months. His CEA level has increased to 54.4, but he still has no radiographic evidence of disease, except for a positive positron-emission tomography scan in his abdominal nodes more than 20 months after initiation of vaccination. Patient no. 15 has pseudomyxoma peritonei with metastatic disease in her lungs and abdomen, including measurable disease in her abdominal wall. After having stable disease for 6 months, she had a minor reduction in the size of her abdominal nodes and a decrease in her CEA level when IL-2 was added to her regimen. Her carcinoma progressed in her lungs only and she was taken off study after 10 cycles on therapy. Patients no. 1, 2, and 19 were on study for a long period, but each had no evidence of disease at the beginning and were found to have experienced disease progression on therapy after 8, 10, and 10 months, respectively.

Immunologic Responses

T-cell assays using the HLA-A2–binding CEA agonist peptide (CAP-1-6D) and Flu matrix peptides were used to investigate T-cell responses in patients positive for the

Table 3. Immunologic Results From HLA-A2+ Patients Randomized to Receive VAAA

Patient No.	Treatment	Flu Peptide	CEA Peptide	% Change
4	Pretreatment	1/150,000	1/200,000	229
	VAAA	1/53,846	1/87,500	
5	Pretreatment	1/75,000	1/200,000	328
	VAAA	1/85,000	1/61,000	
14	Pretreatment	1/42,857	1/200,000	307
	VAAA	1/53,000	1/65,000	
15	Pretreatment	1/50,000	1/200,000	500
	VAAA	1/31,000	1/40,000	
18	Pretreatment	1/58,333	1/200,000	230
	VAAA	1/63,000	1/87,000	
19	Pretreatment	1/53,000	1/116,000	+333
	VAAA	1/36,000	1/35,000	

HLA-A2 allele. Ficoll-purified PBMCs from each of these patients were purified and viably frozen at approximately 1×10^7 cells/mL. PBMCs were obtained prevaccination and 4 weeks after each vaccination cycle for each patient. The ELISPOT assays, using the CEA and Flu peptides and PBMCs from each patient prevaccination and 4 weeks after each vaccination cycle, were performed simultaneously and coded. PBMCs were assayed after only 24 hours in culture in the presence of peptide to rule out effects of in vitro selection of T-cell populations. Results were expressed as precursor frequency of IFN-γ–secreting cells in response to the given peptide; a higher number of precursors is expressed by a smaller number in the denominator of the precursor frequency. As seen in Table 3, responses to the Flu matrix 9-mer peptide were quite similar before and after the vaccinations. These data, and the use of an aliquot (from frozen vials) of PBMCs from a normal donor and the Flu peptide, also served as an internal control for the ELISPOT assay.

Given the results of the ELISPOT analysis, an increase in CEA-specific T-cell precursor frequencies was observed in six of six patients in the VAAA cohort (Table 3) compared with two of five patients in the AAAV cohort (Table 4) after four vaccination cycles. For example, the CEA-specific T-cell precursor frequency of PBMCs obtained from patient no. 15 (VAAA) was one in 40,000 after four vaccinations, compared with less than one in 200,000 before vaccination. A statistical comparison of the two cohorts (see Patients and Methods) revealed a statistically significant ($P < .01$) increase in CEA-specific T-cell precursor frequencies for patients in the VAAA cohort when compared with patients in the AAAV cohort (Fig 1). The VAAA cohort exhibited an average increase of 217.8% in CEA-specific T-cell precursor frequencies, whereas the AAAV cohort exhibited only a 48.0% increase. Only minor differences in immune responses to the Flu matrix peptide were observed post- versus prevaccination (8.2% increase in VAAA cohort *v* 24.2% decrease in AAAV cohort); both were not statistically significant ($P = .12$).

In addition to the patients described above, there were two patients (nos. 10 and 11) who received three cycles of avipox-CEA in a previous clinical trial.[19] Both patients showed statistically significant CEA-specific T-cell responses after four additional cycles of vaccinations (VAAA). This finding suggested that a delay in vaccinations neither improved nor degraded the anti-CEA T-cell immune response. It should be pointed out that patients no. 10 and 11 were not included in the above calculations. One patient (no. 13) was removed from the trial after one vaccination, and T-cell responses were not analyzed.

Table 4. Immunologic Results From HLA-A2+ Patients Randomized to Receive AAAV

Patient No.	Treatment	Flu Peptide	CEA Peptide	% Change
2	Pretreatment	1/9,677	1/60,000	−75
	AAAV	1/15,152	1/79,800	
12	Pretreatment	1/19,354	1/150,000	+140
	AAAV	1/38,000	1/107,142	
16	Pretreatment	1/42,000	1/200,000	257
	AAAV	1/50,000	1/77,700	
20	Pretreatment	1/87,500	1/200,000	None
	AAAV	1/77,000	1/200,000	
21	Pretreatment	1/116,000	1/200,000	None
	AAAV	1/120,000	1/200,000	

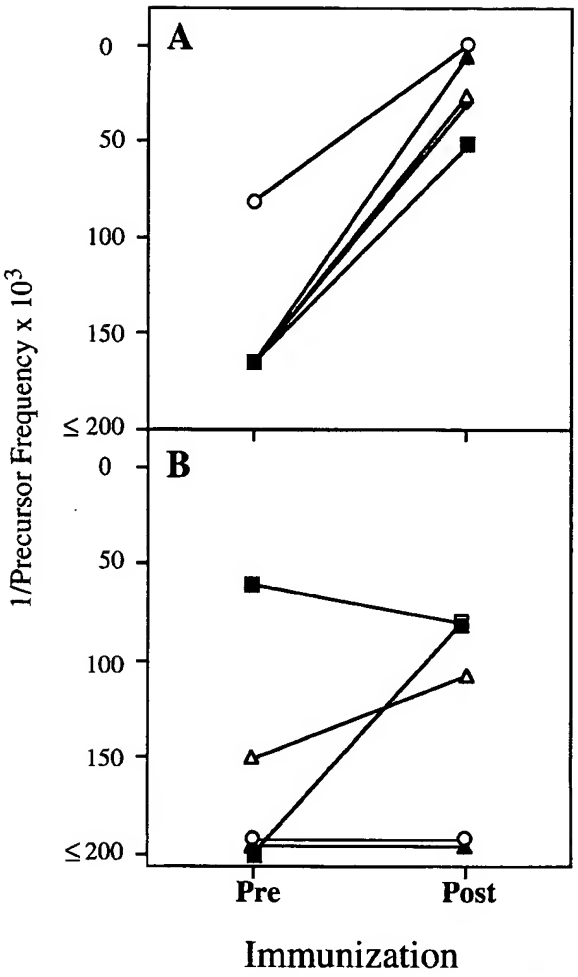

Fig 1. Changes in CEA-specific T-cell precursor frequencies before the first vaccination (pre) and after 4 vaccinations (post) from patients treated with VAAA (A) and AAAV (B). The ELISPOT assay using the CEA peptide and IFN-γ production was used.

Antibody Assay Results

All patients treated on this trial were analyzed to determine whether they produced antibodies directed against CEA. Four patients have shown such a result: results from two of these patients are depicted in Figs 2 and 3. Serum from patient no. 21 (Fig 2) showed no reactivity in Western blot analysis to either native CEA or recombinant CEA, using prevaccination serum. Postvaccination serum from this patient showed IgG reactivity to both CEA preparations in Western blot. No reactivity was seen to the control antigen BSA using either pre- or postvaccination serum. Interestingly, patient no. 15 had preexisting antibodies to native CEA (Fig 3). However, after four vaccinations, this patient was found to have increased IgG antibody against

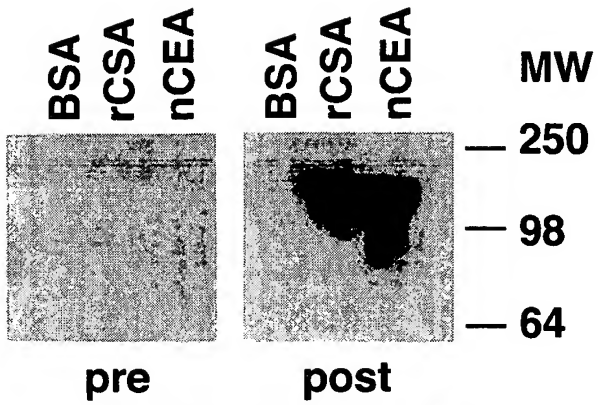

Fig 2. Induction of CEA-specific IgG responses after vaccination. CEA-specific IgG in serum (1:100 dilution) from patient no. 21 pre- (left) and postvaccination (right) versus native CEA (nCEA), recombinant CEA (rCEA), and BSA (control).

native CEA as well as reactivity against recombinant CEA. No reactivity was seen to the control antigen BSA using either pre- or postvaccination serum (Fig 3).

The Role of Cytokines

Of the nine patients with stable disease who elected to continue vaccinations by receiving avipox-CEA in combination with GM-CSF, six were HLA-A2–positive and, therefore, could be immunologically monitored using the ELISPOT technique. The results of the assays from five patients who received cytokines are shown in Figs 4 (VAAA cohort) and 5 (AAAV cohort). All five patients exhibited an increase in CEA-specific T-cell precursor frequency after the first vaccination cycle in which GM-CSF was introduced. Note that although CEA-specific precursors continued to increase, Flu-specific precursors remained the same (Fig 4). The results from Fig 4 also demonstrate that avipox-CEA can be given at least four times with resulting increases in CEA-specific T cells. As

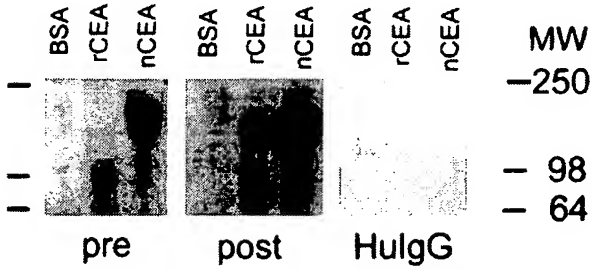

Fig 3. Induction of CEA-specific IgG responses after vaccination. CEA-specific IgG in serum (1:40 dilution) from patient no. 15 pre- and postvaccination. Each panel contains 3 lanes that include purified nCEA, rCEA, and BSA (control). (right) Results with normal human IgG (HulgG).

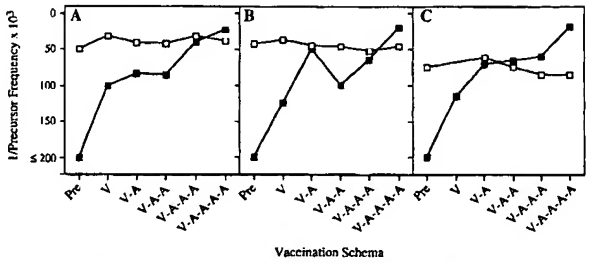

Fig 4. The impact of GM-CSF on T-cell responses. Patients no. 15 (A), 14 (B), and 5 (C) received rV-CEA (V) followed by 3 vaccinations with avipox-CEA (A), all without cytokine. At the fifth vaccination (VAAAA), all patients received avipox-CEA with recombinant GM-CSF. Depicted are T-cell responses to the 9-mer Flu peptide (□) and to the 9-mer CEA peptide (■) using the ELISPOT assay.

seen in Fig 5B, patient no. 2 (AAAV cohort) did not respond to the initial four cycles of vaccinations (Table 4); however, he showed a 651% increase in T-cell precursor frequency after the first cycle of treatment with avipox-CEA in combination with GM-CSF (Fig 5B). Similar results were also observed in patient no. 21, who did not respond after the initial four cycles (Table 4) but showed a 733% increase in CTL precursor frequency after two cycles of avipox-CEA in combination with GM-CSF (Fig 5A). As seen in Figs 4A through 4C and Fig 5B, T-cell precursors to Flu remained the same, whereas CEA-specific precursors increased with additional vaccinations. At this time, it is not known why Flu precursors also increased after the fifth vaccination of patient no. 21 (Fig 5A), because the internal control of simultaneous assay to Flu from a normal donor was consistent to all other assays. Perhaps a Flu infection or increase in precursors owing to GM-CSF was responsible.

CEA-specific T-cell precursor frequencies continued to increase after additional vaccinations of avipox-CEA in combination with GM-CSF in the majority of patients. Three HLA-A2–positive patients elected to be treated with additional vaccinations by receiving avipox-CEA in combination with GM-CSF and IL-2. T-cell precursor frequencies in all three patients continued to increase after IL-2 was added to the vaccinations, but not to the degree that they had after the addition of GM-CSF. It was not clear whether this response was mediated by IL-2 or by the additional treatments with GM-CSF. However, this result was not observed in a previous clinical trial in which patients continued to receive avipox-CEA alone (without cytokines) for more than 10 cycles (data not published). As seen in Fig 5B, patient no. 2 demonstrated a marked increase in CEA-specific T-cell precursors (and not Flu precursors) after the fifth vaccination (AAAV followed by A in the presence of GM-CSF). Although not as marked, CEA-specific precur-

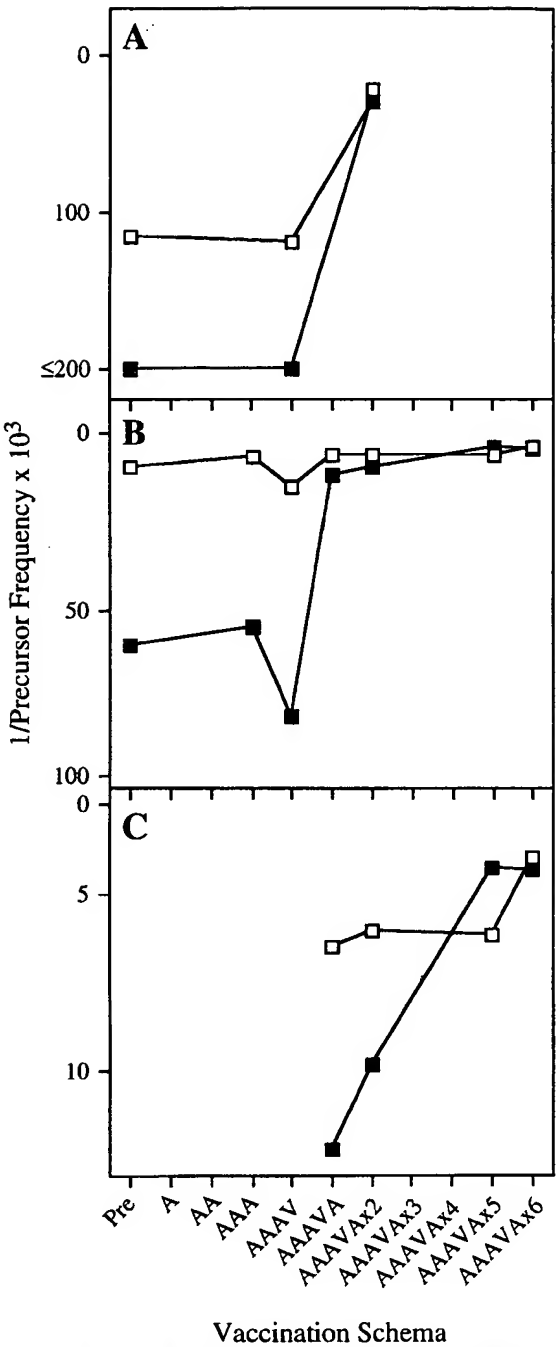

Fig 5. The impact on CEA-specific T-cell responses of multiple vaccinations of avipox-CEA escalated with the addition of GM-CSF and IL-2. Patients no. 21 (A) and 2 (B and C) received the AAAV vaccination regimen without the addition of cytokine. (A and B) No enhancement in T-cell responses for Flu (□) or CEA (■) after 4 vaccinations. Vaccinations no. 5 and 6 with avipox-CEA (A) also included GM-CSF. Vaccinations no. 7 through 9 were with avipox-CEA and GM-CSF followed by low-dose IL-2. Vaccination no. 10 consisted of avipox-CEA with GM-CSF. (C) Expanded scale of (B) showing results postvaccination 5 through 10 for patient no. 2.

sors of this patient continued to increase after nine vaccination cycles. This is demonstrated in the expanded scale of Fig 5B shown in Fig 5C; here, it can be seen that CEA-specific T-cell responses to the CEA peptide continued to increase through nine vaccinations, including eight vaccinations with avipox-CEA.

DISCUSSION

This phase I study demonstrates for the first time the safety in humans of a diversified prime-and-boost vaccination protocol using recombinant vaccinia virus and recombinant nonreplicating avipox virus. This study also compared, for the first time, two diversified vaccination schedules (VAAA *v* AAAV) by monitoring the level of CEA-specific T-cell precursors in HLA-A2–positive patients. On the basis of a statistical analysis of the two vaccination schedules, VAAA was concluded to be the more effective dose schedule. This result, however, must be evaluated considering variations in the patient population comprising each study group. Variations in tumor burden (four of six patients in the AAAV cohort had evidence of metastatic disease compared with three of six patients in the VAAA cohort), tumor size, primary tumor site, lowered immune status, and/or immune suppression may have been confounding variables in this comparison of the two dose schedules, although no such variations were found (Table 2). We thus conclude that VAAA remains the preferable dosage schedule over AAAV because VAAA produced positive CEA-specific T-cell responses in all six patients assayed, whereas AAAV showed responses in only two of five patients. Furthermore, the VAAA cohort showed increased average T-cell responses ($\geq$ 328%) compared with the AAAV cohort (approximately 80% increase). These studies validate, for the first time, a diversified prime-and-boost vaccination protocol in patients.

This study also began to investigate the effects of local GM-CSF and low-dose IL-2 when administered after vaccination with avipox-CEA. It seems that both of these cytokines were effective in increasing the frequency of CEA-specific T-cell precursors in all six HLA-A2–positive patients assayed. However, it cannot be determined at this time whether the increase in CEA T-cell responses is due to either the addition of cytokines, additional vaccinations, or both. Patients are currently being accrued to the second stage of this study, in which the safety and efficacy of GM-CSF and IL-2 during the initial four cycles of vaccinations are being investigated. Patients no. 2 and 21 (both in the AAAV cohort), who did not respond immunologically to the initial four cycles of vaccinations, showed marked responses after GM-CSF was added to the vaccinations. The planned phase I/II study investigating the safety and efficacy of GM-CSF and IL-2 will more conclusively test the benefit of including low-dose IL-2 in these treatments.

The use of this diversified prime-and-boost vaccination protocol is not limited to the 50% of the population that is positive for HLA-A2. Although immunologic monitoring was conducted for patients who were HLA-A2–positive for proof of concept, these vaccines can potentially elicit T-cell responses in patients of any other HLA type, because CEA peptides have already been identified that elicit cytolytic T-cell responses in vitro for HLA-A24, HLA-A3, and other alleles.[10,24-27] Efforts to expand the number of monitoring tools are ongoing. Antibody responses were also observed in some patients on this study; this, of course, could give an additional measure of CEA-specific immune responses in HLA-A2–negative patients, as well as suggest stimulation of the humoral arm of the immune system by these vaccines. Nonetheless, the ELISPOT assay proved to be quite effective in measuring CEA-specific T-cell immune responses, and there is the suggestion that clinical responses may mirror immune responses in some patients (patient no. 15 and others are now in the stage II portion of the trial).

Despite measurable CEA-specific T-cell responses in patients enrolled in this study, no objective anticancer effects were observed. The reason remains unclear at this time but may be related to the hypothesis that some patients with advanced cancer are unable to respond to immunologic therapy because of lowered immune status and/or preexisting immune suppression. The lowered immune status of cancer patients has been demonstrated through a decrease in the ζ chain of the T-cell receptor and by a shift from a type 1 T-cell response to a type 2 T-cell response. The presence of putative immune inhibitors, such as transforming growth factor beta or IL-10, may be responsible for the immune suppression observed in cancer patients.[28-30] Another explanation for the lack of clinical efficacy is that the number or affinity of T cells generated by the vaccines, given no preexisting immune suppression or lowered immune status, may not have been sufficient to elicit a measurable reduction in tumor size and/or progression. More potent vaccine strategies, such as the incorporation of a triad of costimulatory molecules[27] into the vectors used here and the insertion of the CEA enhancer agonist epitope[16,17] into these vectors, are examples of such planned innovations. Finally, the size and/or high interstitial pressures of tumor masses may have prevented T cells from penetrating the tumor(s). Subsequent studies will try and define the role of tumor burden on the ability of patients to generate an immune response.

We have reported here for the first time the use of a diversified prime-and-boost vaccination protocol using two

different recombinant vectors in humans and have validated that the VAAA dose schedule is preferable to AAAV for use in future studies. Moreover, these studies have demonstrated, for the first time, that avipox-CEA can be given up to eight times with continued increases in CEA T-cell responses. These studies thus form the rational basis for the use of diversified prime-and-boost vaccine strategies in less advanced disease settings.

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Induction of p53-specific Immune Responses in Colorectal Cancer Patients Receiving a Recombinant ALVAC-p53 Candidate Vaccine[1]

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ABSTRACT

Purpose: **The tumor-associated auto-antigen p53 is commonly overexpressed in various types of human cancer, including colorectal cancer. Experiments in preclinical models have shown that it can serve as a target for T-cell-mediated tumor-eradication. The feasibility of a p53-specific therapeutic vaccination was investigated in cancer patients.**

Experimental Design: **A Phase I/II dose-escalation study was performed that evaluated the effect of a recombinant canarypoxvirus (ALVAC) vaccine encoding wild-type human p53 in 15 patients with advanced colorectal cancer. Each group of five patients received three i.v. doses of one-tenth of a dose, one-third of a dose, or 1 dose of the vaccine [1 dose = $1 \times 10^{7.5}$ cell culture infectious dosis $(CCID)_{50}$].**

Results: **Potent T-cell and IgG antibody responses against the vector component of the ALVAC vaccine were induced in the majority of the patients. Enzyme-linked immunosorbent-spot assay (ELISPOT) analysis of vaccine-induced immunity revealed the presence of IFN-γ-secreting T cells against both ALVAC and p53, whereas no significant interleukin-4 responses were detected. Vaccine-mediated enhancement of p53-specific T-cell immunity was found in two patients in the highest-vaccine-dose group.**

Conclusions: **This study demonstrated the feasibility, even in patients with advanced cancer, to elicit immune responses against the ubiquitously expressed tumor-associated auto-antigen p53. Our results form the basis for additional studies that will explore the antitumor capacity of p53 containing multivalent vaccines in cancer patients with limited tumor burden.**

INTRODUCTION

Tumor antigens such as CEA[3] (1–3), epithelial cell adhesion molecule (Ep-CAM; Refs. 4 and 5), and p53 (6–8) represent potential targets for the immunotherapy of colorectal cancer. Mutations in the *p53* tumor suppressor gene are found in a wide variety of tumors, including ~50% of colorectal cancers (9–11). Because p53 is not expressed at the cell surface, p53-specific antibodies are unlikely to exert therapeutic antitumor effects. In contrast, p53-specific T-cell immunity may be exploitable for immunotherapy of cancer because p53-peptides are processed by the proteasome and presented by MHC class I molecules to CTLs (12–15). Furthermore, accumulated p53, when released from dying tumor cells, can serve as a potent immunogen for Th-cells. Both p53-specific MHC class I-restricted CTLs and p53-specific MHC class II-restricted Th-cells have been shown to exert antitumor efficacy *in vivo* in mouse tumor models (8, 13, 16, 17). Importantly, these experiments demonstrated that p53-specific immunity was not accompanied by overt signs of autoimmunity.

In humans, p53-specific antibodies have been found in patients suffering from a variety of tumors (18–23). The induction of anti-p53 antibodies generally reflects a high tumor load and is, therefore, associated with bad prognosis. wt.p53-specific CTLs (12, 24–26) and Th-cells (27) have been detected in human PBMC cultures *in vitro*. In addition, wt.p53-specific proliferative responses were demonstrated in patients with breast cancer (28) and for several years after resection in the majority of patients with resected primary colorectal cancer (29).

Immunization with recombinant poxvirus carrying a transgene that encoded the tumor antigen of choice can result in strong T-cell immunity against this antigen (30). Given its host-cell specificity, the canarypoxvirus ALVAC can infect a wide array of mammalian cells, including human cells, although

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[3] The abbreviations used are: CEA, carcinoembryonic antigen; APC, antigen-presenting cell; CCID, cell culture infectious dosis; ELISPOT, enzyme-linked immunosorbent-spot (assay); HLA, human leukocyte antigen; IL, interleukin; MRM, memory response mix; PBMC, peripheral blood mononuclear cell; PHA, phytohemaglutinin; Th-cell, T-helper cell; wt.p53, wild-type p53 (protein); mEU, milli-ELISA unit(s); SI, stimulation index.

failing to replicate in such cells. The safety profile of ALVAC-based recombinants has been established in numerous animal models (31). In addition, recombinant ALVAC has been administered to more than 2000 humans, including cancer patients, with no signs of pathological reactions, dissemination of virus into the environment, or viral replication (31). Furthermore, canarypox recombinants effectively prime both the humoral and cellular components of the immune system against the transgene-encoded antigens (32) and, thus, are attractive vehicles for therapeutic vaccination in cancer. Immunization with ALVAC-encoding murine wt.p53 as well as human wt.p53 (ALVAC-hup53, designated vCP207) protected BALB/c mice from a challenge with a highly tumorigenic mouse fibroblast tumor cell line expressing high levels of mutant p53 (33). Optimal triggering of p53-specific T-cell immunity was found when ALVAC was injected i.v., after which it was primarily localized in the lung, liver, and spleen (34). To study the effect of i.v. ALVAC-hup53 injection with respect to general safety and the induction of autoimmunity in nonhuman primates, rhesus macaques were given three i.v. injections of ALVAC-hup53 at proportional doses up to 10-fold higher than those proposed for humans. Repeated administration of the highest doses was well tolerated and despite the >95% amino-acid identity between human and rhesus p53, no abnormalities were detected in hematological or clinical chemistry parameters or tissue pathology that could point to autoimmune reactions. One of four monkeys injected with the maximal dose proposed for humans developed a p53-specific antibody response (35).

To assess the safety and immunogenicity of ALVAC-hup53 in humans, a Phase I/II dose-escalating study was initiated in which 15 end-stage colorectal cancer patients were vaccinated three times i.v. After vaccination, strong humoral and cellular immune responses to ALVAC were induced. Importantly, p53-specific T-cell immunity was induced in several of the patients receiving the highest vaccine dose.

PATIENTS AND METHODS

Clinical Protocol. Adult patients (ages >18 years) with histologically proven colorectal cancer and evidence by imaging techniques of irresectable disease were eligible for inclusion in this study. Patients with metastatic disease that was untreatable by conventional therapies or patients with metastatic disease that was potentially treatable, but who refused conventional therapy were also eligible for this study. The protocol used in this study was approved by the local and national medical ethics committee as well as by the biological safety committee and the Dutch Ministry of Health and Environment. Inclusion criteria were: use of effective contraception; aspartate aminotransferase/alanine aminotransferase levels within three times the normal range; alkaline phosphatase levels within five times the normal range; billiburin levels and blood cell counts within 1.5 times the normal range; serum CEA level of ≥10 μg/liter; and a health status corresponding to the WHO performance status level of 0 or 1. Additionally, at least 30% of the primary tumor or metastases were to express HLA class I and p53 by immunohistochemistry. Exclusion criteria were: pregnancy; autoimmune disease; symptomatic viral or other infections; HIV seropositivity or refusal to hear the results of the HIV test; receipt of organ grafts; life expectancy of <3 months; a history of allergy; a history of severe neurological, cardiovascular, renal, hepatic, endocrine, respiratory. or bone marrow dysfunction; known family history of Li-Fraumeni syndrome; known allergy to egg proteins or neomycin; chemotherapy or radiation within the 4 weeks preceding enrollment; immunotherapy, chemotherapy using nitrosourea; hormonal treatment (other than contraception) within the previous 6 weeks; or a history of treatment with growth hormone extract.

Patients were divided into three groups of five individuals each. Patients received three i.v. injections of ALVAC p53 at three-week intervals. Group 1 received one-tenth of the total dose ($10^{6.5}$ $CCID_{50}$) of ALVAC hup53 at each injection, Group 2 received one-third of the total dose ($10^{7.0}$ $CCID_{50}$) at each injection, and Group 3 received the total dose ($10^{7.5}$ $CCID_{50}$) at each injection. Blood was obtained for biochemical, hematological, and immunological assays before each vaccination. Patient visits (V) were scheduled as follows: (*a*) preinclusion visit (PV) at a maximum of 2 weeks before the first vaccination; (*b*) V1, week 0, first vaccination; (*c*) V2, week 3, second vaccination; (*d*) V3, week 6, third vaccination; (*e*) V4, week 7; (*f*) V5, week 8; (*g*) V6, week 14; and (*h*) V7, week 20. PBMCs collected before vaccination (PV) and 2 weeks after completing the immunization scheme (V5) were isolated, cryopreserved using a computer-controlled freezing device, and stored in liquid nitrogen. Sera were isolated from blood collected at each visit and stored at −20°C.

PBMCs and sera of anonymous healthy blood donors were isolated and used as control PBMCs in ELISPOT and proliferation assays or as negative controls in p53 antibody subtype ELISA.

Recombinant ALVAC-p53 Vaccine. ALVAC-hup53 (vCP207) is a recombinant virus, based on the canarypoxvirus-based vector ALVAC and the wild-type *p53* gene. The vCP207 recombinant was generated by cotransfection of ALVAC-infected primary chick-embryo fibroblasts with an insertion plasmid and noninfectious purified ALVAC genomic DNA, leading to the integration of the foreign gene expression cassette into the viral genome via homologous recombination. The clinical lot used in these studies was produced by Aventis Pasteur (Marcy l'Etoile, France) and was purified twice through a sucrose gradient.

Antigens. Twenty-four peptides spanning the wt.p53 protein were synthesized as 30-mers overlapping by 14 amino acids. These peptides were divided into three pools: pool 1, peptide 1 (p1) to p8 (covering residues 1–142); pool 2, p9-p16 (residues 129–270); and pool 3, p17-p24 (residues 257–393). Recombinant baculovirus-derived human wt.p53 and gp100 protein were produced at Virogenetics, (Troy, NY). Inactivated ALVAC virus was donated by Dr. C. Blondeau (Aventis Pasteur, Marcy l'Etoile, France). MRM, a mixture of tetanus toxoid (150 limus flocculentius/ml; National Institute of Public Health and the Environment, Bilthoven, the Netherlands), *Mycobacterium tuberculosis* sonicate (2.5 μg/ml; generously donated by Dr. P. Klatser, Royal Tropical Institute, the Netherlands) and *Candida albicans* (0.005% HAL Allergenen Lab, Haarlem, the Netherlands) was used to control the capacity of PBMCs to proliferate in response to typical recall antigens.

Antibody Titers. ALVAC-specific IgG antibodies were measured in a standard ELISA. Briefly, inactivated ALVAC virus particles were coated at a concentration of 1 μg/ml After blocking with PBS/BSA 1%, serial dilutions (1:100 to 1:3200) of preimmune and postimmunization sera and a positive-control working standard serum titrated from 130 to 0.2 mEU/ml were applied in duplicate wells. After an incubation period of 2 h at 37°C, wells were washed and incubated with antihuman IgG-horseradish peroxidase for another 2 h at 37°C. Color was developed with ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate); Sigma Chemical Co.] + 0.0075% H_2O_2. The absorbance at 415 nm was measured. The anti-ALVAC antibody titer (mEU/ml) in each serum sample was calculated for each dilution using a linear regression curve obtained by plotting the working standard concentration *versus* the absorbance. The mean titer of all serum dilutions was calculated. Anti-ALVAC titers <1000 mEU/ml were considered negative.

p53-specific antibodies were measured as reported previously (29). Briefly, microtiter wells were coated overnight at 4°C with 100 μl of recombinant baculovirus-derived p53 or, as a control, BSA at a concentration of 2 μg/ml. Wells were washed and then incubated with PBS + 1% powdered milk (Protifar; Nutricia, the Netherlands). After 1 h of incubation at 37°C, the wells were washed, and patient-derived serum or a positive control were diluted 100 times in PBS+1% powdered milk, and 100 μl was added to the wells for 2 h at 37°C. Subsequently, the wells were washed and incubated for 1 h at 37°C with horseradish peroxidase-labeled second antibody-specific for human immunoglobulin, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgM, or IgE (Southern Biotechnology Associates, ITK, Uithoorn, the Netherlands) diluted in PBS+1% powdered milk. After extensive washing, ABTS substrate was added. After 30 min of incubation, the absorbance was measured at 415 nm. As a control, sera obtained from three healthy blood donors were tested in each assay for each antibody isotype. Patient sera with an absorbance higher than the mean absorbance of the three-donor sera + two times the SD, were considered positive. In addition, p53-specific IgG titers were quantified using the commercially available p53-ELISA of Pharmacell (IM 2208; Immunotech, Marseille, France). The anti-p53 IgG titer (units/ml) of each serum sample was calculated at 100 × dilution using a linear regression curve obtained by plotting the standard concentration *versus* the absorbance. Results were interpreted as follows: negative, <0.9 units/ml; equivocal, 0.9–1.1 units/ml; and positive, ≥1.1 units/ml.

Lymphocyte Stimulation Test. Pre- and postimmunization PBMCs were seeded in a 96-well U-bottomed plate at 150,000 cells/well and incubated with 5 μg/ml of indicated antigens, MRM at a 1:50 dilution, or PHA in six to eight replicate wells. PBMCs, stimulated with PHA, were seeded in a separate plate. At day 6, PBMCs were pulsed with 0.5 μCi [^{3}H]thymidine (5 Ci/mmol; Amersham, Aylesbury, United Kingdom) per well for 18 h. Plates were harvested with a Microcell Harvester (Skatron, Lier, Norway). Filters were packed in plastic bags containing 10 ml of scintillation fluid and subsequently counted in a 1205 Betaplate counter (Wallac, Turku, Finland). The PHA controls were pulsed with 0.5 μCi [^{3}H]thymidine at day 4 and harvested the next day. The mean SI was calculated by dividing the mean of the experimental wells by the mean of medium control wells. SI ≥ 4 were considered positive (29).

Table 1 Patient characteristics

	All patients	Group 1 (n = 5)	Group 2 (n = 5)	Group 3 (n = 6)
Gender				
Male	12	3	5	4
Female	4	2	0	2
Age (yr)				
Mean	60	53	62	65
Range	42–71	42–66	46–69	57–71
Diagnosis (mo)				
Mean	25	35	14	25
Range	6–66	17–66	7–21	6–56
Therapy				
First line chemotherapy	15	5	5	5
Second line chemotherapy	7	4	2	1
ILP[a]	8	4	1	3
Other	8	2	1	5

[a] ILP, isolated liver perfusion.

Analysis of Antigen-specific T-Cells by ELISPOT. The ELISPOT assay was performed as reported previously (36). Pre- and postimmunization PBMCs were analyzed for the production of both IFNγ and IL-4. Briefly, PBMCs were seeded at a density of 2.5 × 10^6 cells/well of a 12-well plate (Costar, Cambridge, MA) in 1 ml of ISCOVE's medium (Life Technologies, Inc.) enriched with 10% FCS, in the presence or absence of indicated pools of p53 peptide (5 μg/peptide/ml), inactivated ALVAC (5 μg/ml), or MRM at a 1:50 dilution. After 4 days of incubation at 37°C, PBMCs were harvested, washed, and seeded in six replicate wells at a density of 10^5 cells/well of a Multiscreen 96-well plate (Millipore, Etten-Leur, the Netherlands) coated with an IFNγ-catching antibody or an IL-4-catching antibody (Mabtech AB, Nacha, Sweden). Pre- and postimmunization PBMCs, stimulated with the same antigen, were seeded in adjacent wells. The ELISPOT was further performed according to the instructions of the manufacturer (Mabtech). The number of spots was analyzed with a fully automated computer-assisted video imaging analysis system (Carl Zeiss Vision). Specific spots were calculated by subtracting the mean number of spots + 2 × SD of the control (medium) from the mean number of spots of experimental wells. Results were expressed as the number of specific spots above the lower detection limit of the assay (10 cells/10^6 PBMCs) per million PBMCs.

Statistical Analysis. To compare the proliferative responses either to common recall antigens (MRM) or to PHA of patient-derived PBMCs *versus* PBMCs derived from healthy blood donors, the mean SIs were calculated, log-transformed, and compared in a Welch-corrected unpaired t test. The nonparametric ANOVA Kruskal-Wallis test was used to compare different vaccination doses with the maximal anti-ALVAC antibody titer or the number of postimmunization ALVAC-specific T cells in the ELISPOT. The maximal anti-ALVAC antibody titer in patients was correlated with preexisting ALVAC-specific T-cell frequencies using Spearman rank correla-

Table 2 Evaluation of the immunological patient status

	T-cell response to recall antigens						PHA	
	7-day proliferation[a]		IFNγ ELISPOT[b]		IL-4 ELISPOT		3-day proliferation[c]	
Patient no.	PV	V5	PV	V5	PV	V5	PV	V5
2[d]	7	7	1540	810	70	—	43	28
3	23	NT[e]	1260	60	280	NT	237	NT
4	18	16	890	440	30	50	71	39
5	12	40	230	560	10	30	70	417
6	NT	NT	420	—	170	20	NT	NT
7	7	—	250	—	—	—	61	18[f]
8	76	72	2340	3770	—	—	439	212
9	44	78	200	290	—	—	107	114
10	14	24	1120	240	—	30	82	203
11	—	10	50	410	—	—	130	185
12	10	105	120	1600	90	180	751	470
14	—	—	—	—	—	—	318	316
15	—	—	200	120	—	60	4.5[f]	14
16	34	—	—	—	NT	NT	267	489
17	11	12	410	380	40	—	118	975

[a] PBMCs were stimulated with the recall antigens containing MRM in eight replicate wells for 7 days, after which [^{3}H]thymidine incorporation was measured. The numbers shown are the SIs of the sample taken before immunization (PV) and 2 weeks after the last immunization (V5). SI ≥ 4 are regarded positive; —, SI < 4.

[b] PBMCs stimulated for 4 days with MRM were harvested, counted, and seeded in ELISPOT plates in order to quantify the number IFNγ or IL-4 producing T cells. Figures indicate the number of antigen-specific cytokine-producing cells per 10^6 PBMCs. —, the number of antigen-specific T cells is below the detection level of the assay.

[c] PBMCs were cultured for 3 days in the presence of PHA, after which [^{3}H]thymidine incorporation was measured. The numbers shown are the SIs. SIs ≥ 4 are regarded positive; —, SI < 4.

[d] Patients 1 and 13 were not completely vaccinated and were excluded from the analysis.

[e] NT, not tested because of unavailable PBMCs.

[f] High background proliferation of the medium control.

tion. Analysis was performed using GraphPad InStat (GraphPad Software Inc.).

RESULTS

General Immune Status of Patients. Sixteen patients were enrolled in the trial (Table 1), 15 of whom completed the immunization scheme. These 15 patients were analyzed for preexisting and vaccine-induced immunity.

Vaccination of end-stage cancer patients may fail to elicit immunity attributable to the presence of large tumor burdens that can result in immunosuppression as reflected by loss of reactivity to common recall antigens (37). In view of these considerations, we tested this recall response before and after the vaccinations were given. With the exception of patient 14, all of the patients showed a response to the mixture of common bacterial antigens at the start of the study. When the vigor of proliferation to the recall antigens of patients was compared with that found in healthy controls (not shown), both the mean proliferative responses (mean SI, 19 and 86, respectively; $P = 0.001$, Welch corrected t test) and the mean number of IFNγ-producing T cells (mean, 695 and 1506. respectively; $P = 0.04$, Welch corrected t test) of the patients were significantly lower, indicating partially suppressed cellular immunity. At the end of the study, patients 6, 7, and 16 lost reactivity to the recall antigens (Table 2). PBMCs of all of the patients responded well to the polyclonal stimulus PHA (Table 2), and, although the mean proliferative responses of patients were lower compared with the responses noted in 13 random healthy controls (mean SI, 207 and 409, respectively), this difference was not significant ($P = 0.2$, Welch corrected t test).

Vaccine-induced Immunity against the ALVAC Vector. To monitor the impact of the vaccine on the immune system, the response to ALVAC was measured. Both antibody and T-cell responses against ALVAC were found to be strongly increased after vaccination in all but one patient (Table 3 and Fig. 1*A*). The T-cell response in patient 5, who also failed to generate an anti-ALVAC antibody response, was only marginal compared with the responses against ALVAC in the other patients. ALVAC-specific T cells mainly produced IFNγ on recognition. IL-4-producing T cells were detected at considerably lower levels and only in the patients that had received the highest vaccine dose. Before vaccination, low levels of IFNγ-producing ALVAC-specific T-cell immunity were detected in 12 of the patients (Table 3) and in 3 of 5 healthy control donors (not shown), which indicated that this phenomenon is quite common. The number of preexisting ALVAC-specific T cells was strongly correlated with higher antibody titers after completion of the immunization scheme ($P = 0.02$, Spearman rank correlation, $r = 0.6$; Table 3).

Vaccine-induced p53-specific Antibodies. The presence of p53-specific IgG antibodies in the sera of all of the patients was tested by a quantitative p53-specific IgG ELISA. In 10 patients, p53-specific IgG antibodies were detected after vaccination, two of which were relatively high titered (patient 6, 7 units/ml; patient 9, 14 units/ml). In three of these patients (patients 4, 7, and 12) no p53-specific IgG antibodies were

Table 3 Humoral and cellular reactivity against ALVAC

Patient no.	ALVAC-specific responses							
	Antibodies[a]		7-day proliferation[b]		IFNγ ELISPOT[c]		IL-4 ELISPOT	
	PV	MAX	PV	V5	PV	V5	PV	V5
2[d]	305	2157 (3)	—	4	—	2230	—	—
3	285	>30,000 (2)	6	NT[e]	160	1650	—	NT
4	277	23464 (5)	4	15	670	1760	—	—
5	583	907 (7)	—	—	—	30	—	—
6	184	1039 (6)	NT	NT	80	750	—	—
7	977	>30,000 (3)	6	—	1910	—	—	—
8	291	11908 (6)	13	104	1290	6800	—	—
9	428	>30,000 (5)	19	67	250	1920	—	—
10	200	8804 (6)	—	10	—	1010	—	—
11	403	4505 (5)	—	39	30	2310	—	30
12	686	>30,000 (4)	9	100	220	3830	—	480
14	353	>30,000 (3)	—	34	50	2960	—	20
15	367	>30,000 (2)	9	16	670	1660	—	190
16	341	26667 (4)	72	179	1530	3880	NT	NT
17	73	>30,000 (3)	27	116	2660	4910	20	20

[a] The pre-existing (PV) anti-ALVAC antibody titer as well as the maximum level (MAX; mEU/ml) is shown. The numbers in parentheses indicate at which visit number this maximum level was reached. An antibody titer <1000 mEU/ml is considered negative.

[b] Inactivated ALVAC at 5 μg/ml was used to stimulate PBMCs. See Table 2, footnote *a*, for additional explanation of 7-day proliferation assay.

[c] See Table 2, footnote *b*, for information on ELISPOT assays.

[d] Patients 1 and 13 were not completely vaccinated and were excluded from the analysis.

[e] NT, not tested because of unavailable PBMCs.

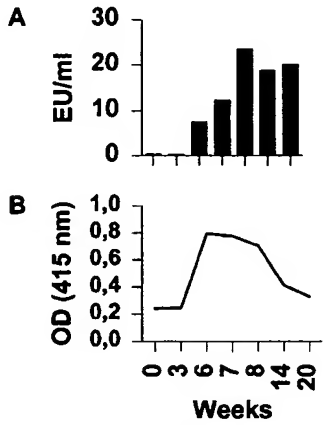

Fig. 1 Longitudinal analysis of the anti-ALVAC IgG response (*A*) and p53-specific IgM (*B*) response of patient 4 before and after vaccination with the lowest dose of ALVAC-hup53. Vaccinations were given at weeks 0, 3, and 6. *OD*, absorbance.

detected before vaccination, which implies that these responses were induced by the vaccine (Table 4). Not unexpectedly, the other seven patients with advanced colorectal cancer had demonstrable p53-specific IgG antibodies before vaccination. These responses were, however, not boosted by the vaccine (Table 4). In two patients (patients 4 and 10), it was noted that the p53-specific IgM level was increased after one or two immunizations (Fig. 1B, and data not shown). Thus, in a fraction of advanced colorectal cancer patients, vaccination with ALVAC-hup53 results in the induction of p53-specific antibodies.

Vaccine-induced p53-specific T-Cell Immunity. After vaccination with the one-tenth or one-third dose of ALVAC-hup53, no induction of p53-specific proliferation or p53-specific cytokine production (ELISPOT, detection limit of 10 cells per 10^6 PBMCs; Table 4) was detected. Importantly, ELISPOT analysis of the PBMC samples of patients injected with the full dose of ALVAC-hup53 showed an IFNγ-producing p53-peptide-specific response in four of five patients. No p53-specific IL-4 production was detected. Patient 12 responded to peptide pool p9-p16 and p17-p24, patients 15, 16, and 17 responded to p9-p16 with T-cell frequencies ranging from 60 to 190 per million PBMCs in the postimmunization samples. In patients 16 and 17, these responses were also detected before vaccination (Table 4). Furthermore, the p53-specific response of the latter patient was also detected in the less sensitive lymphocyte-stimulation proliferation assay. In conclusion, our data demonstrated that injection of end-stage colorectal cancer patients with $10^{7.5}$ $CCID_{50}$ (full dose) of ALVAC-hup53 can induce p53-specific T-cell immunity.

DISCUSSION

Immunotherapy approaches against cancer that target self-antigens aim at the induction of a beneficial autoimmune response. Although the observation that p53 is commonly overexpressed in many cancer types makes it an attractive target antigen, the feasibility of p53-specific immunotherapy is challenging because this unique antigen is ubiquitously expressed in normal tissues. As such, immunological tolerance as well as p53-specific immunity to normal cells could preclude the use of vaccines aiming at the induction of p53-specific T-cell immunity. Notably, experiments in mice have indicated that p53-

Table 4 Humoral and cellular p53-specific reactivity

Patient no.	p53-specific responses													
	Antibodies[a]		7 day proliferation[b]						IFNγ ELISPOT[c]					
			PV			V5			PV			V5		
	PV	MAX	p1–p8	p9–p16	p17–p24	p1–p8	p9–p16	p17–p24	p1–p8	p9–p16	p17–p24	p1–p8	p9–p16	p17–p24
2[d]	3.6	3.6 (1)	—	—	—	—	—	—	—	—	—	—	—	—
3	1.4	1.5 (3)	—	—	—	NT[e]	NT	NT	—	—	—	—	—	—
4	—	1.1 (4)	—	—	—	—	—	—	—	—	—	—	—	—
5	1.1	1.2 (3)	—	—	—	—	—	—	—	—	—	—	—	—
6	7.4	11.9 (5)	NT	NT	NT	NT	NT	NT	—	—	—	—	—	—
7	0.9	1.1 (4)	—	—	—	—	—	—	—	—	—	—	—	—
8	1.1	2.6 (6)	—	—	—	—	—	—	—	—	—	—	—	—
9	14.4	14.4 (1)	—	—	—	—	—	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	—	—	—	—	—	—	—	—
12	0	1.6 (3)	—	—	—	—	—	—	—	—	—	—	70	70
14	—	—	—	—	—	—	—	—	—	—	—	—	—	—
15	—	—	—	—	—	—	—	—	—	—	—	20	190	—
16	—	—	—	—	—	—	—	—	—	40	—	—	60	—
17	1.5	2.7 (4)	—	4	—	—	5	—	20	470	—	60	200	—

[a] The pre-existing (PV) anti-p53 IgG antibody titer as well as the maximum level (MAX; units/ml) is shown. Interpretation: negative, <0.9 units/ml; equivocal, 0.9–1.1 units/ml; positive, ≥1.1 units/ml. The numbers in parentheses indicate at which visit number this maximum level was reached.

[b] PBMCs were stimulated with three different pools of 30 amino-acid-long overlapping p53 peptides containing either peptide 1 to peptide 8 (p1–p8), peptide 9 to 16 (p9–p16), or peptide 17 to 24 (p17–p24) at a concentration of 5 μg/ml per peptide. See Table 2, footnote *a*, for additional information on 7-day proliferation.

[c] See Table 2, footnote *b*, for information on ELISPOT assays.

[d] Patients 1 and 13 were not completely vaccinated and were excluded from the analysis.

[e] NT, not tested because of unavailable PBMCs.

specific immunotherapy may be feasible. In view of these findings, we have conducted a Phase I study involving p53-specific vaccination of end-stage colorectal cancer patients. Antibody responses were induced in some, but not all, of the vaccinees. Moreover, p53-specific IFNγ-producing T-cell immunity was found to be induced in two of five colorectal cancer patients vaccinated with the highest dose of $10^{7.5}$ $CCID_{50}$ ALVAC-hup53. The present study demonstrates that even in a group of end-stage cancer patients, with a less than fully competent immune system, immunization with ALVAC-hup53 results in the induction of p53-specific B-cell and T-cell immunity. Furthermore, the administration of multiple injections was well tolerated, and no clinical signs of autoimmunity were observed.[4] This is a promising result paving the way for future vaccine trials in patients with less advanced tumor stages, *e.g.*, using the vaccine as adjuvant therapy after cancer surgery with curative intent, to control minimal residual disease or to prevent recurrence.

Both humoral and cellular anti-ALVAC responses were induced in all but one patient. No clear relationship between the vaccine dose and the anti-ALVAC IgG response ($P = 0.07$) or the number of postimmunization ALVAC-specific T cells as detected by IFNγ ELISPOT ($P = 0.1$) was found. Furthermore, the number of postimmunization ALVAC-specific T cells did not correlate with the maximal ALVAC-IgG level. Interestingly, preexisting ALVAC-specific T-cell immunity was detected in both healthy donors (not shown) and colorectal cancer patients. A likely explanation is that this natural response is caused by cross-reactivity with vaccinia virus, to which most of our patients have been exposed in the past. When the maximal anti-ALVAC IgG level was evaluated in relation to the magnitude of the preexisting ALVAC-specific T-cell response, a clear correlation ($P = 0.01$) was found suggesting that the presence of ALVAC-specific T cells provided help to boost the humoral response to ALVAC. There was no correlation between ALVAC-immunity and the induction of p53-specific immunity.

At first sight, the frequency of the preexisting p53-specific IgG antibody responses in our patient group (47%) seems higher than generally reported in literature (25%; Ref. 18). The induction of p53-specific antibodies is dependent on a subset of mutations in p53 and overexpression of these mutated p53 proteins (reviewed in Ref. (38). Normally, about 50% of colorectal cancer patients display p53 overexpression (39), but, as part of the inclusion criteria, all of the vaccinated patients show overexpression of p53 in the tumor. In line with this, p53-specific antibody responses were more frequently observed in this patient group than in general.

Our assays, in which peptide pools of 30-residue-long peptides were used, highly favor the detection of p53-specific

[4] A. G. Menon, P. J. K. Kupper, S. H. van der Burg, R. Offringa, M. C. Bonnet, B. I. J. Harinck, R. A. E. M. Tollenaar, A. Redeker, H. Putter, P. Moingeon, H. Morreau, C. J. M. Melief, and C. J. H. van de Velde. Safety of intravenous administration of a canarypox virus encoding the human wild-type *p53* gene in colorectal cancer patients, submitted for publication.

T-helper responses (29, 36). In this Phase I/II trial, four of five patients receiving the highest dose of ALVAC-hup53 displayed p53-specific Th-cells, which, on recognition of a p53 peptide, produced IFNγ but not IL-4. Although p53-specific immunity was induced in two patients receiving the highest dose of the vaccine, the other two patients displayed p53-specific Th-cells already before immunization. Previously, it has been shown that p53-specific Th-cells can arise during tumor growth in mice (40) and humans (28, 29). In fact, our study in patients treated for primary colorectal carcinoma by surgery showed that the majority of these patients had developed a p53-specific Th-response (29). Our current data suggest that p53-specific Th-immunity is less frequently detected in end-stage colorectal cancer patients who failed conventional treatment such as surgery and extensive chemotherapy treatments. Altogether, our data show that p53-specific Th-cells either can develop as part of the natural immune response in tumor-bearing patients or can be induced by p53-specific vaccination (*e.g.*, patients 12 and 15). Not unexpectedly, p53-specific T-cell responses were not accompanied by significant clinical responses. The end-stage patients had large tumor burdens that were diagnosed 25 months, on average, before vaccination, and the tumor response was evaluated 14 weeks after completing vaccinations.

A key question concerns the antitumor efficacy of these p53-specific Th-cells and the necessity to boost these responses by a vaccine. Cumulative evidence has shown that tumor-specific CD4+ Th-cells are pivotal for the efficient eradication of solid tumors, although such tumors usually do not express MHC class II (reviewed in Ref. 6). In several murine tumor models, tumor-specific CD4+ Th-cells critically contributed to the development and efficacy of antitumor responses (41–46). In two cases, Th-cells were shown to exert their antitumor effect by stimulating tumoricidal macrophages and eosinophils (43, 44), whereas, in the other cases, tumor-specific Th-cells drove the CTL-dependent antitumor immunity. The efficacy of these Th-cells lies not only in the property of providing CTLs with essential growth stimuli, primarily IL-2, during the effector phase (47) but also in the ability to deliver essential activation signals to APCs needed for an optimal priming of tumor-specific CTLs (48–51). Notably, whereas the delivery of this type of T-cell help to CTLs requires the APCs to present both tumor-derived MHC class I-restricted CTL epitopes and tumor-derived MHC class II-restricted Th-epitopes, these epitopes do not have to be derived from the very same antigen (41). Recently, vaccination with recombinant ALVAC virus expressing the CEA, which is also associated with colorectal cancer, resulted in the induction of CEA-specific CTLs. Of note, both p53-specific and CEA-specific T-cell frequencies were of the same magnitude (this study and Refs. 1 and 52). This implies that if APCs present in the tumor-draining lymph node take up both p53 and CEA, the p53-specific Th-response may be used to provide a license to kill for CTLs against CEA or to other colorectal cancer-associated antigens.

At present, three different colorectal cancer-associated tumor antigens: p53, CEA, and epithelial cell adhesion molecule are evaluated for use in vaccines to treat colorectal cancer. Vaccination with each of these antigens has proven to be safe and to result in the specific induction of T-cell immunity (1–7). The use of a combinatorial vaccine comprising all three of these antigens is, therefore, highly desirable. In view of the above, a follow-up trial, involving the immunization of cancer patients with limited disease, with a vaccine comprising all three antigens is currently being initiated.

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Amplification of Virus-Induced Antimelanoma T-Cell Reactivity by High-Dose Interferon-α2b: Implications for Cancer Vaccines

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ABSTRACT

Purpose: **The therapeutic effectiveness of cancer vaccines, composed of tumor antigens that are also self-antigens, may be limited by the normal mechanisms that preserve immunological tolerance. Consistent with this notion, we found that vaccination of melanoma patients with recombinant viral vaccines expressing gp100 (a melanoma antigen also expressed by normal melanocytes) produced only transient increases in noncytotoxic T cells specific for immunodominant gp100 epitopes. To improve the therapeutic effects of these vaccines, IFN-α2b (IFN-α) was administered to some high-risk patients.**

Experimental Design: **7 HLA-A*0201$^+$ patients were injected with high doses of IFN-α (20 MU/m^2 × 20 doses) at various times after completing the vaccination protocol. Clinical toxicity and responses were documented, and the effects on gp100-reactive T cells were measured by IFN-γ enzyme-linked immunospot assays, tetramers of HLA-A*0201 and gp100 epitopes, and cellular cytotoxicity assays.**

Results: **In patients who had previously responded to vaccination, high doses of IFN-α recalled gp100-reactive T cells with the ability to kill gp100-expressing tumor targets *in vitro*. Concomitant with the reappearance of these cytotoxic T cells, tumor regression was observed in the two patients with clinically evident metastatic disease.**

Conclusions: **The finding that high-dose IFN recalls previously activated tumor-reactive T cells with potent killing ability suggests a strategy to maintain antitumor responses initiated by cancer vaccines.**

INTRODUCTION

Cancers such as melanoma that are incurable with conventional chemotherapy (1) may be susceptible to vaccines that enhance the activity of tumor-reactive T cells (2). A number of tumor antigens have been identified and used to make specific cancer vaccines. For melanoma, these antigens include members of the MAGE family, tyrosinase, melanA/Mart-1, Trp-2, and gp100 (3–5).

Despite the identification of these target antigens, current vaccines often activate T cells for only a short time without providing strong antitumor activity (6). Possible explanations for this transient activation include: (*a*) T cells are often only weakly reactive to tumor antigens that are also self-antigens; (*b*) T cells exposed to these antigens during tumor progression may become anergic; (*c*) immunoregulatory mechanisms that prevent sustained autoimmune responses may also inhibit antitumor responses; and (*d*) tumor cells, alone, may not be able to sustain the vaccine-primed antitumor responses (6, 7).

We recently evaluated the effects of viral gp100-based vaccines in melanoma patients with skin or lung metastases or at high risk of developing metastatic disease because of deep primary lesions or LN[3] involvement (8). In agreement with others (6), we found that vaccine-induced anti-gp100 T-cell responses were often transient (Table 1). In an attempt to provide additional therapeutic benefits, we administered HDI to some of these patients because survival of such patients can be prolonged by HDI for 1 month followed by low doses of IFN-α (10 MU/m^2) s.c. for 48 weeks (9). However, the major therapeutic effect of IFN-α may be provided by the high-dose component because low dose therapy, alone, does not produce this survival benefit (9).

We found that HDI could recall antitumor T-cell responses in patients who had previously mounted immune responses to the viral vaccines. In marked contrast to tumor-reactive T cells activated by the vaccines alone, T cells recalled by HDI killed tumor antigen-bearing targets *in vitro* and were associated with evidence of tumor regression *in vivo*. These results lend insight into the mechanism of action of IFN-α in cancer therapy and

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[3] The abbreviations used are: LN, lymph node; IFN-α, Interferon-α2b; HDI, high-dose IFN-α; PBMC, peripheral blood mononuclear cell; FLU, Influenza matrix protein (residues 58–66); CTL, cytotoxic T cell; APC, antigen-presenting cell; FU, follow-up; IL, interleukin; ELISPOT, enzyme-linked immunospot.

may have implications for the pathogenesis of autoimmune diseases in general. They also suggest that HDI after vaccination with tumor antigens may be an effective immunotherapeutic strategy.

PATIENTS AND METHODS

Patients. Eligibility criteria included a confirmed diagnosis of melanoma with metastases [or high risk (10) of developing metastases], the HLA-A*0201 haplotype [identified by sequence-specific primer-PCR (11) at the Aventis-Pasteur HLA laboratory], age > 18 years, Eastern Cooperative Oncology Group performance status of 0 or 1, and informed, written consent according to national and institutional guidelines. All patients had previously been vaccinated as part of a Phase I trial sponsored by Aventis-Pasteur.

Aventis Vaccine Trial. The vaccine used in this trial involved the ALVAC (2)-gp100M recombinant virus (12, 13; made from a second generation Canarypox virus expressing a full-length gp100 gene encoding two epitopes modified for enhanced HLA class I binding) along with the two modified peptide epitopes (described below). The purpose of the trial was to evaluate the toxicity of these reagents and also the feasibility and effectiveness of injecting them directly into inguinal LNs under ultrasound guidance. Four groups of 6–8 HLA-A*0201$^+$ melanoma patients (satisfying the criteria described above) were vaccinated using different routes of administration. Patients in group I received ALVAC-gp100 (0.5 ml, 0.5 × 10^7 plaque-forming units/ml) s.c. on day 1 of a 21-day cycle for three cycles followed by 2 ml of a mixture of the modified gp100 peptides (500 μg/ml of each peptide) s.c. on day 1 of a 21-day cycle for two cycles. Patients in group II received 0.4 ml of the gp100 peptide mixture intranodally daily for 5 days every 21 days for 2 cycles. Patients in group III received ALVAC-gp100 s.c. on day 1 of a 21-day cycle for three cycles followed by the gp100 peptide mixture (0.4 ml intranodally daily for 5 days every 21 days for two cycles). Patients in group IV received ALVAC-gp100 (0.5 ml intranodally) on day 1 of a 21-day cycle for three cycles followed by the gp100 peptide mixture (0.4 ml intranodally daily for 5 days) every 21 days for two cycles. Full details of this ongoing trial will be published separately. Of the patients in the study of HDI after vaccination reported in this article, M237 was in group I, M136 and M260 were in group II, M302, M246, and M166 were in group III, and M335 was in group IV.

Treatment with HDI. HDI (Schering Canada, Pointe-Claire, Quebec, Canada) consisted of 20 injections of IFN-α (20 MU/m^2/day) over 4 weeks (14). The IFN-α dose was reduced by 33% for severe toxicity [grade 3 or 4, defined by the common toxicity criteria (version 2.0) established by the National Cancer Institute Cancer Treatment Evaluation Program (15)].

Study Design. Consenting patients still considered at risk for developing progressive disease were administered HDI after completing the Aventis-sponsored vaccine trial. Toxicity was monitored weekly while patients received HDI. Toxicity and disease status (determined by clinical and/or radiological evaluation) were monitored monthly during the 3-month FU period. PBMCs were collected at these times by density gradient centrifugation and kept in liquid nitrogen until used for immunological monitoring.

Reagents. HLA-A*0201-restricted peptide epitopes for CTLs (from Aventis Pasteur, Toronto, Ontario, Canada) included FLU (GILGFVFTL; Ref. 16), gp100 epitopes modified to increase class I MHC binding [gp100:209-2M (IMDQVPFSV) and gp100:280-9V (YLEPGPVTV; 17)], and the HIV p17 Gag protein-derived peptide (SLYNTVATL; Ref. 18). The gp100 peptides (5 mg/ml stock) were dissolved in water and the others (10 mg/ml stock) in DMSO. CD8-FITC antibodies were purchased from PharMingen (San Francisco, CA). Antibodies from BB7.2 [anti-HLA-A2; Ref. 19; obtained from the American Type Culture Collection (Manassas, VA)] were purified and labeled with FITC in our laboratory. Tetramers (20) of HLA-A*0201 complexed to the peptides YLEPGPVTV (Lot No. BL/0757), IMDQVPFSV (Lot No. BL/0755), or GILGFVFTL (Lot No. BL/0839) bound to phycoerythrin-labeled streptavidin were purchased from Pro-Immune Ltd. (Oxford, United Kingdom). T2 cells were from the American Type Culture Collection.

***In Vitro* T-Cell Stimulation.** PBMCs were thawed, washed, and incubated overnight in AIM-V medium (Life Technologies, Inc., Burlington, Ontario, Canada) at 37°C in 5% CO_2. Cells were then adjusted to 2–3 × 10^6 cells/ml in AIM-V plus 5% AB serum (Sigma; complete media) and plated (1 ml/well) in 24-well polystyrene tissue culture grade plates (Becton Dickinson Labware, Franklin Lakes, NJ) with FLU or both gp100 peptides added at previously optimized final concentrations of 10 or 25 μg/ml, respectively. IL-2 (50 IU/ml; Chiron, Emeryville, CA) was added 3 and 6 days later, and the cells were harvested after 8 or 9 days for ELISPOT or cytotoxicity assays.

IFN-γ ELISPOT Assays. ELISPOT assays were performed as previously described (21) with minor modifications. Capture and biotinylated detection antibodies were from the 1-DIK and 7-B6-1 clones, respectively (Mabtech, Stockholm, Sweden). Cultured T cells were reactivated in triplicate wells with FLU or both modified gp100 peptides (final concentrations of 10 and 25 μg/ml, respectively). IL-2 (100 IU/ml) was also included, except in control cultures stimulated by phorbol myristic acetate (20 ng/ml; Sigma) and Ionomycin (1 μg/ml; Calbiochem, San Diego, CA).

Cellular Cytotoxicity. T2 targets (5 × 10^6 cells) labeled with ^{51}Cr (500 μCi; Perkin-Elmer, Boston, MA) were washed and incubated with 10 μg/ml gp100:209-2M and gp100:280-9V (mixed 1:1) or FLU for 2 h at 37°C. Washed targets (5 × 10^3 cells) were cultured with varying numbers of effectors for 4 h, and chromium release assays were then performed as described (22). Total release (TR) was measured by lysis of targets with 10% Triton-X (Sigma), and spontaneous release (SR) was measured without effector cells. Percent-specific lysis was defined as (cpm − SR)/(TR − SR) × 100%.

Immunofluorescence. Staining was performed at the end of the *in vitro* culture period as described previously (22).

RESULTS

Toxicity. Seven HLA-A*0201$^+$ patients (Table 1) received HDI at various times (mean ± SD = 7.2 ± 4.9 months) after a final booster injection of modified gp100 epitopes (17, 23). Patients developed typical toxicities associated with HDI, including flu-like symptoms, cytopenias, and liver function test

Table 1 Patient characteristics

Patient no.	Age (yr)/Sex	Initial disease[a]	Status before HDI	Current status	Time from last vaccine to HDI[b] (mo)	Time from HDI to last FU (mo)	gp100-reactive T-cell frequency before vaccine	Peak gp100-reactive T-cell frequency during vaccine[c]	Peak gp100-reactive T-cell frequency during HDI
M136	52/M	Lung, LN	NED	NED	8	13	$1/7.5 \times 10^4$	$1/5 \times 10^4$	$1/1 \times 10^5$
M302	53/F	Skin metastases	NED	NED	3	16	$1/7.1 \times 10^3$	1/510	1/263
M246	47/F	LN	NED	NED	7	14	$1/1 \times 10^5$	$1/1 \times 10^5$	$1/1 \times 10^5$
M237	49/M	LN	NED	NED	8	14	$1/3.3 \times 10^4$	1/6667	1/1667
M166	33/M	Mesenteric mass	Gluteal mass	Clinical regression	6	11	$1/1.7 \times 10^4$	1/6270	1/1111
M335	32/F	LN, skin, breast	LNs, skin, lung	Clinical regression	1.5	9	$1/4.1 \times 10^4$	1/588	1/667
M260	64/M	LN	Lung	Lung (no change)	17	6	$1/5 \times 10^4$	$1/2 \times 10^4$	$1/1 \times 10^5$

[a] Except for M166 and M335, patients had no evaluable disease (NED) after surgical resections before starting vaccinations. Lung involvement of M260 was documented before commencing HDI.
[b] This period began from the last gp100 peptide injection (as described in the "Patients and Methods" section) and ended at the first infusion of HDI.
[c] Peak frequency was the highest number of spots at any time point during active vaccination. ELISPOT assays were performed as described in "Patients and Methods," and the average of three replicate wells is reported as $1/(\text{average spot number}/10^5 \text{ plated cells})$.

Table 2 Toxicity, treatment delays, and dose reductions in patients receiving HDI after vaccination

	Grade 3[a]	Grade 2	Total
Constitutional symptoms	1/7	3/7	4/7
Vitiligo	0/7	1/7	1/7
Elevated liver function tests	1/7	4/7	5/7
Granulocytopenia/leukopenia	1/7	6/7	7/7
Neurologic toxicity	1/7	1/7	2/7
Dose reduction			7/7
Dose delay			7/7

[a] The delivery of HDI was modified for each patient on the basis of common toxicity criteria (15), with grade 4 being the most severe, necessitating stopping treatment. A 33% reduction of dosage occurred after the first treatment interruption and a 66% reduction from baseline dose occurred after the second. No patients had a third treatment interruption that would also have required removal from treatment.

abnormalities, which lasted only during the time of HDI administration (Table 2). One patient (M160) developed neuropsychiatric symptoms, requiring the institution of antidepressants, which also cleared within a week of stopping HDI. One patient (M335) developed vitiligo around skin deposits of melanoma (described below). Dose reductions and treatment delays attributable to toxicity were required for all seven patients (Table 2), which is higher than the 33% incidence reported for 396 patients treated with HDI, alone, in the E1694 Intergroup trial (15).

Recall of Vaccine-Induced gp100-Reactive T Cells by HDI. The requirement for HLA-A*0201 expression allowed the frequency of gp100-reactive T cells to be measured in both ELISPOT assays for IFN-γ production (21, 24) and by flow cytometry using tetramers of recombinant HLA-A*0201 folded around the modified gp100 peptides (25). No patient had circulating gp100-reactive T cells in these assays before HDI (Fig. 1, "FU" dot plots, Fig. 2, *a* and *c*; data not shown). Increased frequencies ($>1/10^4$ cells) of gp100-reactive T cells had been observed in 4 patients (M302, M237, M166, and M335) at some point during the vaccine protocol (Table 1, column 9 and Fig. 1, "Vaccine" dot plots) but decayed rapidly (Figs. 1, "FU" dot plots, and 2, *a* and *c*; data not shown). In these patients, increased frequencies of gp100-reactive T cells were found again by the second week of HDI (Table 1 column 10; Figs. 1, "HDI" dot plots, and 2, *a* and *c*). Treatment with HDI did not recall gp100-reactive T cells if measurable anti-gp100 responses had never been achieved with vaccination (Table 1, columns 9 and 10; patients M136, M246, and M260). Failure to detect gp100-reactive T cells was not attributable to technical problems associated with cryopreservation and culture conditions because FLU-reactive cells [found in 60–70% of patients because of previous exposure to influenza (16, 26)] could be elicited (Fig. 2, *b* and *d*).

Clinical Responses. All patients completed HDI without disease progression. Two patients (M166 and M335) developed evidence of regression of metastatic melanoma after HDI as described below.

Association of Increased gp100-Reactive T Cells and Clinical Responses after HDI in M166. M166 presented with a 0.6-mm primary skin lesion. A mesenteric metastasis was resected 6 years later. No other metastatic disease was evident until he was considered for the melanoma vaccine study 18

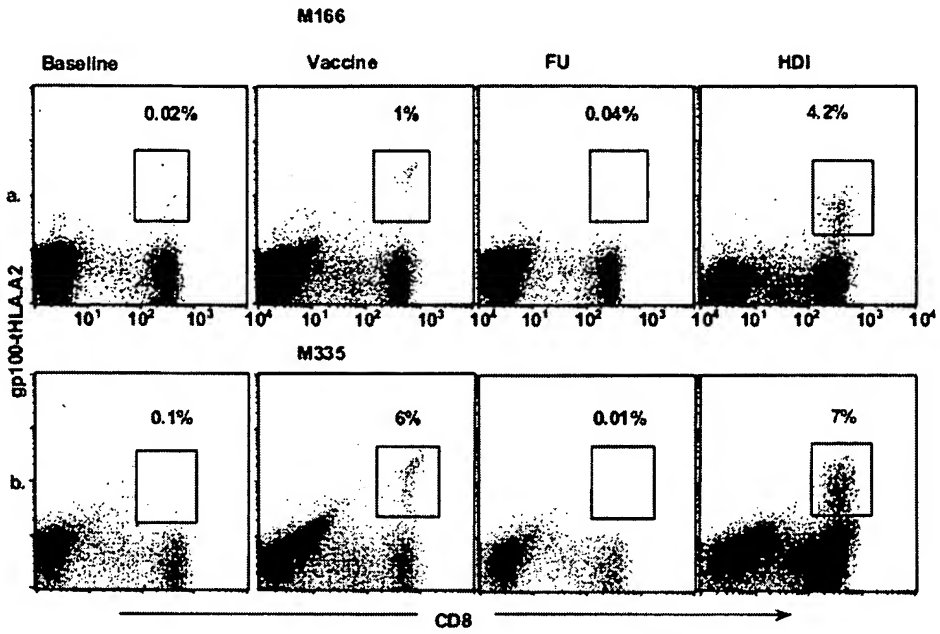

Fig. 1 High-dose IFN-α recalls tumor-reactive T cells previously activated by vaccines. PBMCs from patients M166 (*a*) and M335 (*b*) were stimulated with both HLA-A*0201 binding gp100 peptides as described in "Patients and Methods." The cells were then stained with CD8-FITC antibodies and the two phycoerythrin-labeled gp100 peptide tetramers (labeled "gp100-tHLA-A2" in the figure) and analyzed by flow cytometry. The percentage of $CD8^+$tetramer$^+$ cells (representing gp100-reactive T cells) is indicated in the box in each dot plot. Few gp100-reactive T cells were found before vaccination (*baseline*). Peak responses to vaccination are shown in the dot plots marked "Vaccine." Before HDI, gp100-reactive T-cell numbers had returned to baseline (FU) but increased again after starting HDI (2 and 3 weeks for M166 and M335, respectively).

months later and found to have a gluteal mass (Fig. 3*a*, arrow). The initial bidimensional measurements of this mass (by magnetic resonance imaging) were 2.1 × 3.6 cm and, although it was not biopsied, its appearance was most consistent with a soft tissue metastasis related to melanoma. Moreover, the high T1 signal was consistent with melanin within the mass. A decision was made to observe the mass during vaccination because of the difficult nature of the surgery required for its resection. The mass was somewhat smaller (1.6 × 3.7 cm; Fig. 3*b*, arrow) after active vaccination but improved considerably after HDI (1.1 × 1.2 cm; Fig. 3*c*, arrow) and had not progressed at the last FU visit, 14 months later.

A specific T-cell response was induced in M166 by the gp100-based vaccine (Table 1, Figs. 1*a* and 2*a*). During vaccination, gp100-reactive $CD8^+$ T cells comprised 1% of the total cells in an 8-day culture of PBMCs, primed with gp100:209-2M and gp100:280-9V (Fig. 1*a*, "Vaccine" dot plot). After vaccination, gp100-reactive T-cell frequencies fell (Fig. 2*a*) and disappeared by the time that HDI was instituted (Figs. 1*a*, "FU" dot plot, and 2*a*). One week after HDI, the frequency of IFN-γ-producing gp100-reactive T cells increased to ~1/1000 (Fig. 2*a*) and tetramer-staining $CD8^+$ T cells comprised 4.2% of the culture (Fig. 1*a*, "HDI" dot plot). FLU-reactive $CD8^+$ T-cell frequencies were relatively constant despite HDI and the changing gp100-reactive T-cell frequencies (Fig. 2*b*).

Association of Increased gp100-Reactive T Cells and Clinical Responses after HDI in M335. M335 presented with a 0.65-mm primary skin lesion. Six years later, she developed right inguinal LN involvement that was treated with resection and levamisole (27). Subsequent skin metastases were treated with resection followed by HDI and 10 months of low-dose IFN-α. One year later, she developed a right axillary LN metastasis that was treated with resection and radiation. Shortly thereafter, melanoma recurred in the skin and dermis of the right breast and chest wall and was treated with mastectomy and local radiation. Before vaccination, multiple small melanotic skin metastases covered the right chest without other detectable systemic disease (Fig. 3, *d* and *g*). While actively being vaccinated, she developed a 4-cm mass in the mastectomy scar (data not shown), extensive adenopathy in the cervical region, and left axilla with multiple nodes measuring up to 13 mm (Fig. 3*e*), and multiple lung nodules (the largest being 7 mm; Fig. 3*h*). Although not confirmed by histology, the appearance of these lesions was most consistent with progression of metastatic melanoma. HDI was instituted, leading to rapid improvement of the chest, axillary (Fig. 3*f*), and lung (Fig. 3*i*) metastases. The innumerable small lung nodules became barely discernable, and the 7-mm nodule at the left lung base measured <1 mm. Metastatic nodules remained in the skin but, interestingly, vitiligo had formed around some of them (data not shown), suggesting autoimmune destruction of neighboring melanocytes (28). This patient has been subsequently maintained on s.c., low-dose IFN-α for a year without evidence of additional disease progression.

As was the case with M166, gp100-reactive T cells increased transiently after vaccination but disappeared by the time of initiation of HDI (Figs. 1*b* and 2*c*). However, 2–3 weeks after starting HDI [and concomitant with the clinical response (Fig.

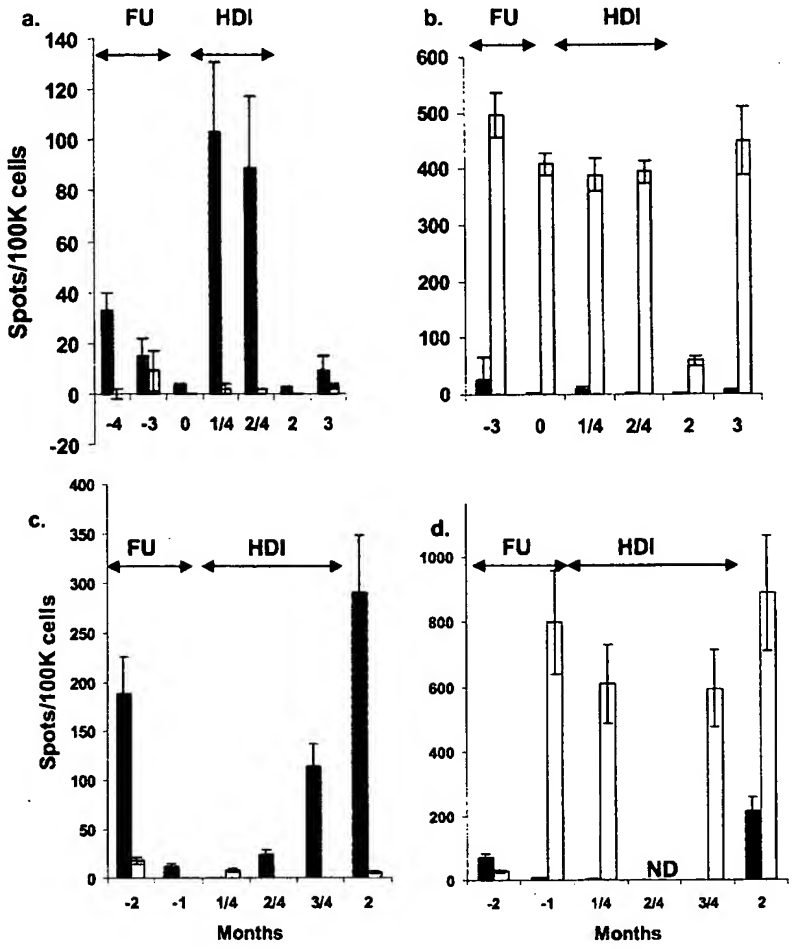

Fig. 2 Increased gp100-reactive T-cell frequencies after HDI. PBMCs were collected from M166 (*a* and *b*) and from M335 (*c* and *d*) during the FU period after active vaccination (indicated by the *double-headed arrow* labeled "FU"), just before beginning HDI, weekly while on HDI (indicated by the *double arrow labeled* "HDI") and then monthly after completion of HDI. The cryopreserved samples were thawed simultaneously and stimulated with the gp100 peptide mixture (*a* and *c*) or FLU peptides (to ensure that the cultures could reveal memory T cells if they were present; *b* and *d*). After 8 days, cells were reactivated on ELISPOT plates with the gp100 peptides (■) or FLU peptides (□). The average and SD of the number of spots from three replicate wells are shown. ND = not done. (Note that the designations "1/4, 2/4, 3/4" refer to the weeks after commencing HDI.)

3)], gp100-reactive T-cell frequencies increased to ~1/667 in the ELISPOT assay (Table 1, column 10; Fig. 2*c*) and tetramer-staining $CD8^+$ T cells increased to ~7% of cultured PBMCs (Fig. 1*b*). Elevated responses in these assays were subsequently maintained for at least 2 months (Fig. 2*c*).

HDI Alters the Quality of the Antitumor T-Cell Response. The differences between the frequencies of gp100-reactive T cells during vaccination and recalled by HDI (Table 1, columns 9 and 10; Figs. 1 and 2, *a* and *c*) did not seem to be of sufficient magnitude to account for the clinical effects observed in M166 and M335. We wondered if the quality of the T cells recalled by HDI had changed to account for these clinical effects. Type 1 responses (that result in activation of CTLs able to kill tumor cells) are generally considered to be required for optimal antitumor immunity (29). Although IFN-γ production is a surrogate marker for CTLs (24), we examined directly the ability of gp100-reactive T cells from M166 and M335 to kill antigen-bearing targets. Because melanoma cell lines from these patients were not available, gp100 peptide-loaded T2 cells were used as targets. T2 cells express complexes of peptides and HLA-A*0201 molecules on their cell surface only when HLA-A*0201 binding peptides are provided because of a defective transporter associated with antigen-processing system (30–32). If gp100-reactive T cells are unable to kill peptide loaded T2 cells, it seems unlikely they could kill autologous melanoma cells with a lower surface density of gp100 peptide-HLA-A*0201 complexes.

Despite similar frequencies of tetramer binding and IFN-γ producing gp100-reactive T cells, there were striking differences in the killing of gp100-peptide-loaded T2 cells before and after HDI. Tumor-reactive T cells activated by vaccination alone were unable to kill gp100 peptide-loaded T2 cells (Fig. 4, graphs "After vaccine"). However, gp100-reactive T cells during and after HDI from both patients were potent killers of gp100 peptide-loaded T2 cells (~80% lysis at an E:T ratio of 10:1; Fig. 4). This level of killing was comparable with that observed at the same time with FLU-stimulated T cells and FLU peptide-loaded T2 targets (Fig. 4, graph "Flu-After vaccine"). Direct addition of IFN-α to the cultures did not increase gp100-specific CTL activity (data not shown).

DISCUSSION

In this study, we have shown that HDI alters both the quantity and the quality of autoreactive T cells that recognize

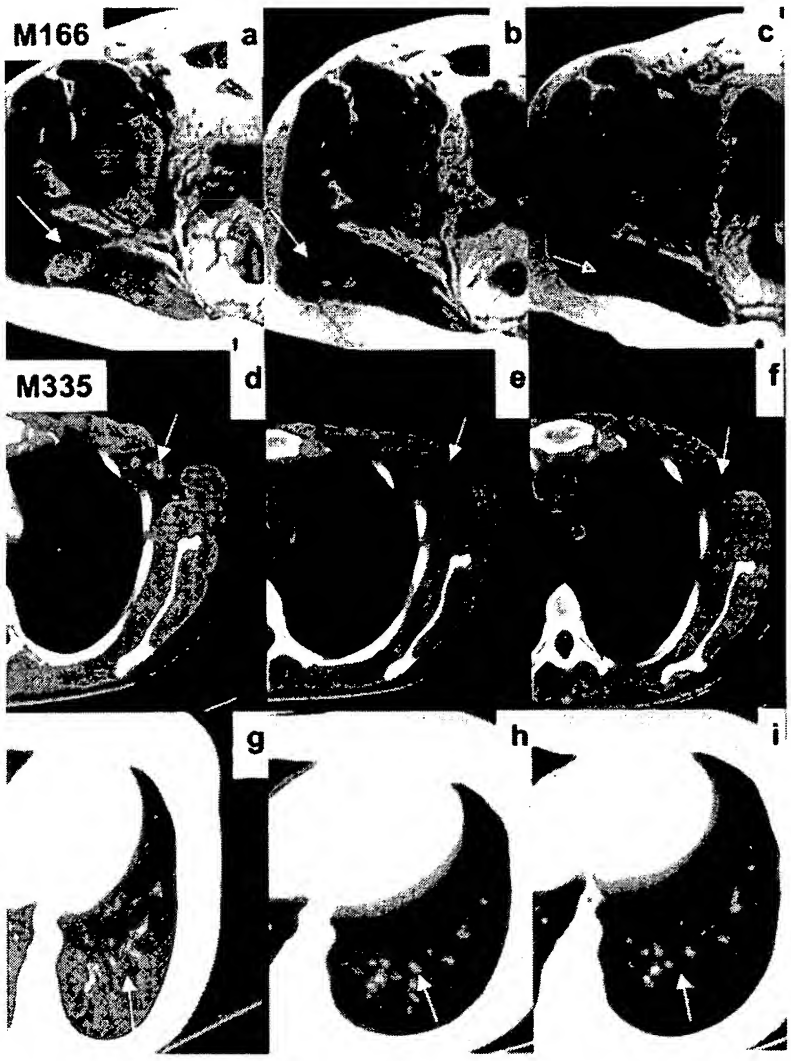

Fig. 3 Clinical responses to HDI. M166: magnetic resonance imaging studies of a gluteal mass (*arrows*), presumed on clinical and radiological grounds to be metastatic melanoma, before vaccination (bidimensional measurements of 2.1 × 3.6 cm; *a*), 3 months after completing the vaccination protocol (1.6 × 3.7 cm; *b*), and 1 month after completing HDI (1.1 × 1.2 cm; c). The mass was marginally smaller after the vaccination protocol but showed considerable improvement after HDI. M335: computerized axial tomography scans of left axillary adenopathy (*d–f*; *arrows*) and a lung nodule (*g–i*; *arrows*) before vaccination (*d* and *g*), 1 month after completing vaccination (*e* and *h*), and 1 month after completing HDI (*f* and *i*). Both areas of involvement progressed through active vaccination but regressed considerably after HDI.

tumor antigens. Specifically, both the number of melanoma-reactive T cells and their ability to kill tumor targets were increased by the administration of HDI after vaccination with viruses that expressed gp100.

As a single agent, IFN-α is thought to inhibit melanoma cell proliferation by directly regulating gene expression (9). IFN-α may also affect antigen presentation by increasing MHC expression on both melanoma cells and professional APCs (33). The effects on vaccine-induced antitumor responses, described here, suggest that IFN-α may also act by recalling the responses of T cells that have been naturally activated by tumor antigens.

How HDI increases the number of vaccine-induced tumor-reactive T cells is not entirely clear. Increased presentation of gp100 antigens—directly by residual melanoma cells or indirectly by professional APCs—could restimulate recently vaccine-activated gp100-reactive T cells. Alternatively, IFN-α could prevent the death of gp100-reactive T cells that are chronically activated by residual melanoma cells (34) and increase their numbers (35). In mice, IFN-α causes antigen-independent proliferation of $CD8^+$ T cells (36) by stimulating IL-15 production from stromal cells (37). Similar effects in humans could cause the reappearance of recently vaccine-activated T cells in the blood and would be consistent with the fact that we could not mimic the *in vivo* results by directly adding IFN-α to cell cultures.

The more potent CTL responses observed *in vitro* after HDI (Fig. 4) were mirrored in the clinical responses of the patients. M335, especially, had suffered disease progression after both single agent IFN-α and during vaccination but showed evidence of tumor regression when HDI was administered after vaccination (Fig. 3). It is not clear how the tumor-reactive T-cell population that arose during vaccination was altered by HDI to promote more effective killing of tumor cells. Simple activation of the cellular lytic machinery by IFN-α (38) is unlikely to account for the phenomenon because increased killing was noted 8 days after the cells had been removed from exposure to IFN-α *in vivo*. Moreover, the addition of IFN-α to *in vitro* cultures was not able to induce specific killing by noncytotoxic gp100-reactive T cells (data not shown).

As a result of the treatment *in vivo* with IFN-α, the tumor-

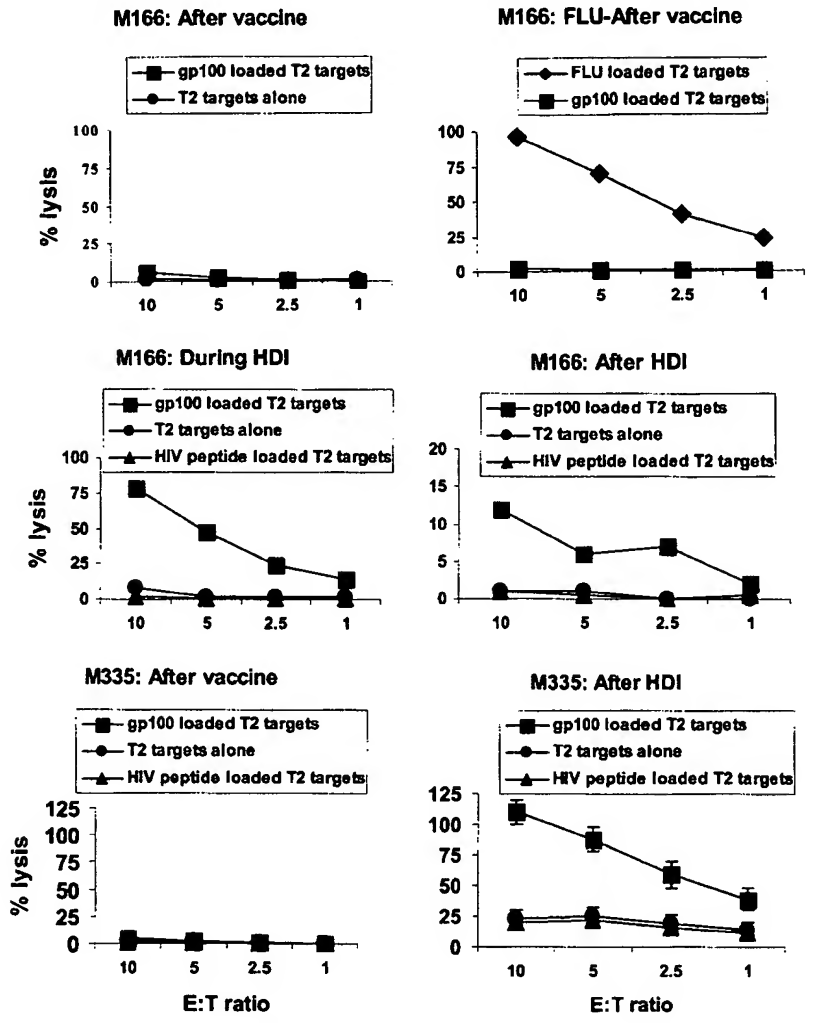

Fig. 4 Enhanced killing activity of gp100-reactive T cells during HDI. Cryopreserved PBMCs after vaccination, during HDI, and 1 month after HDI from M166 (*top and middle rows*) and after vaccination and 1 month after HDI from M335 (*bottom row*) were stimulated with gp100:209-2M and gp100:280-9V for 8 days. The cells were then harvested and the proportions of $CD8^+tetramer^+$ cells from each of the cultures were determined by flow cytometry. Equivalent numbers of $CD8^+tetramer^+$ cells from each culture were then incubated with ^{51}Cr-labeled T2 cells that had been coated with gp100 or control peptides. The ratios of $CD8^+tetramer^+$ effector cells to targets are indicated on the *X* axis. The average and SD of the percent lysis from four replicate wells is shown. Specific killing of gp100 peptide-coated tumor targets was only seen when patients had received HDI. The graph marked "FLU-After vaccine" shows the CTL activity against FLU peptide-coated T2 cells when the same PBMCs from M166 had been activated by FLU peptides and indicates that the culture conditions could support specific CTL activity if it was present.

reactive T-cell population appeared to become dominated by T cells with sufficiently strong reactivity against tumor antigens to mediate CTL activity. This change in the character of the responding T-cell population may be analogous to affinity maturation of an immune response (39) or result from competition between different gp100-reactive T-cell clones for effective activation by the IFN-α-treated APCs (40). Alternatively, only the most potent gp100-reactive T cells may survive the effects of inflammatory cytokines induced by IFN-α, as we recently described for T cells differentially activated by superantigens (41).

Appropriate surrogate markers are important for monitoring the effectiveness of cancer vaccines (42). IFN-γ ELISPOT assays and flow cytometric enumeration of defined tumor antigen-reactive T cells with peptide-MHC tetramers (43) are often used as surrogate markers, as in this study. However, the observation that the quality of the tumor-reactive T-cell population was clearly different after HDI [despite similar frequencies of gp100-reactive T cells in ELISPOT and tetramer assays (Table 1 and Fig. 1, respectively)] suggests that additional studies are required to properly gauge the results of a clinical vaccine. Killing assays (Fig. 4), using autologous tumor cells as targets, would seem to be an excellent assay to gauge these results.

The observations reported in this article suggest that HDI may improve the effectiveness of clinical cancer vaccines by both focusing the responses induced by vaccines onto potent CTLs able to kill tumor cells and maintaining the duration of activity of these cells. The optimal dose and timing of IFN-α remain to be determined. It is possible that lower doses of IFN-α [with only modest activity as a single agent (9)] may be more effective as a vaccine adjuvant. Similarly, the patients who mounted recall responses to gp100 (M302, M237, M166, and M335) began HDI 3, 8, 6, and 1.5 months after their last vaccination, respectively (Table 1), whereas the patients who did not (M136, M246, and M260) began HDI 8, 7, and 17 months later. Although these numbers of patients are too small to draw definite conclusions about the timing of IFN-α, it would seem logical to prescribe it fairly soon after completing a vaccination protocol.

Our observations may also have bearing on the well-known

association of autoimmune diseases with infections (44). In the same way that strong autoimmune responses to gp100 (45) occurred after a viral vaccine and HDI, endogenous IFN-α produced in response to natural infections (33) may sometimes lead to sufficient activation of autoreactive T cells to cause clinically evident autoimmunity.

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Monoclonal Anti-MAGE-3 CTL Responses in Melanoma Patients Displaying Tumor Regression after Vaccination with a Recombinant Canarypox Virus[1]

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We have analyzed the T cell responses of HLA-A1 metastatic melanoma patients with detectable disease, following vaccination with a recombinant ALVAC virus, which bears short *MAGE-1* and *MAGE-3* sequences coding for antigenic peptides presented by HLA-A1. To evaluate the anti-MAGE CTL responses, we resorted to antigenic stimulation of blood lymphocytes under limiting dilution conditions, followed by tetramer analysis and cloning of the tetramer-positive cells. The clones were tested for their specific lytic ability and their TCR sequences were obtained. Four patients who showed tumor regression were analyzed, and an anti-MAGE-3.A1 CTL response was observed in three of these patients. Postvaccination frequencies of anti-MAGE-3.A1 CTL were 3×10^{-6}, 3×10^{-3}, and 3×10^{-7} of the blood CD8 T cells, respectively. These three responses were monoclonal. No anti-MAGE-1.A1 CTL response was observed. These results indicate that, like peptide immunization, ALVAC immunization produces monoclonal responses. They also suggest that low-level antivaccine CTL responses can initiate a tumor regression process. Taken together, our analysis of anti-MAGE-3.A1 T cell responses following peptide or ALVAC vaccination shows a degree of correlation between CTL response and tumor regression, but firm conclusions will require larger numbers. *The Journal of Immunology,* 2003, 171: 4898–4904.

The "cancer-germline" genes, such as the *MAGE* gene family, are expressed in male germline cells and not in other normal tissues. They are also expressed in many tumors of various histological types (1–3). These genes code for Ags that can be recognized on tumor cells by T lymphocytes, and these Ags ought to be strictly tumor specific because the only normal cells that express the encoding genes, the spermatogonia, do not bear HLA molecules on their surface. A large number of MAGE antigenic peptides have been identified, that are recognized on human tumors by HLA class I- or class II-restricted T cells (4–7).

Ags encoded by gene *MAGE-3* have been used for small-scale therapeutic vaccination trials of melanoma patients with detectable disease. The vaccines consisted of either an antigenic peptide, a protein, or dendritic cells pulsed with an antigenic peptide (8–11). In the peptide and protein trials, tumor regressions were observed in ~20% of the patients (Refs.9 and 10 and our unpublished data), a proportion which appears to be well above the rates of spontaneous regressions that have been reported (12). In the absence of a randomized study including a placebo control arm, which could demonstrate vaccine effectiveness, our working hypothesis is that the regressions are caused by the vaccine.

The failure of 80% of the vaccinated patients to show tumor regression could be due to two major causes which are not mutually exclusive: a failure of the vaccine to induce an adequate T cell response or a resistance of the tumor to immune attack. If a limiting factor for success is the level of the T cell response to the vaccine, one ought to find a correlation between the occurrences of T cell responses and those of tumoral regression.

We focused our efforts on the detection of CTL recognizing the antigenic peptide MAGE-$3_{168-176}$.A1, which is encoded by gene *MAGE-A3* and presented by HLA-A1. To detect the anti-MAGE-3.A1 CTL, our approach is based on an in vitro restimulation of PBMC with the antigenic peptide over 2 wk, followed by labeling with A1/MAGE-3 tetramers. To evaluate precursor frequencies, these mixed lymphocyte-peptide cultures (MLPC)[3] are conducted under limiting dilution conditions. Cells that are labeled with the tetramer are cloned and their diversity is analyzed by TCR sequencing (13).

To strengthen the basis of our evaluation of low-level CTL responses, we have recently considered the size of diversity of the anti-MAGE-3.A1 TCR repertoire (Refs. 13 and 14 and C. Lonchay, S. Lucas, T. Boon, and P. Van Der Bruggen, manuscript in preparation). Our latest estimate of the frequency of naive anti-MAGE-3.A1 T cells found in the blood of individuals without cancer is $\sim 4 \times 10^{-7}$ of the CD8 cells. In a noncancerous individual, we obtained a series of 14 independent anti-MAGE-3.A1

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[3] Abbreviations used in this paper: MLPC, mixed lymphocyte-peptide culture; HS, human serum; CTLp, CTL precursor.

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CTL clones, all expressing different TCR. On this basis, we conclude that it is very likely that the TCR diversity of the naive anti-MAGE-3.A1 repertoire is larger than 40, implying a frequency below 10^{-8} for each naive clonotype. Assuming this, there is a lower than 5% probability of finding in a naive repertoire the same CTL clone three times in a set of <10 clones or four times in a set of <20 clones. On the basis of these considerations, we conclude that a patient has made an anti-MAGE-3.A1 CTL response if either the CTL precursor (CTLp) frequency is $\sim 4 \times 10^{-6}$ of CD8 or if "repeated" CTL clones are found in at least the proportions just mentioned.

We have applied the MLPC/tetramer/cloning method to a metastatic melanoma patient who responded clinically to vaccination with peptide MAGE-3.A1 administered without adjuvant (15). We found that the vaccination induced at least a 100-fold amplification of a single anti-MAGE-3.A1 CTL clone, with a postvaccination frequency of 5×10^{-5} of the CD8 cells. This blood frequency was confirmed by a direct evaluation using clonotypic PCR. In another patient who showed tumor regression after vaccination with peptide MAGE-3.A1, we observed a CTL response that was also monoclonal (13).

We describe here the CTL responses of a number of metastatic melanoma patients following vaccination with a recombinant canarypox virus of the ALVAC type containing a minigene encoding the MAGE-1.A1 and MAGE-3.A1 peptides (Refs. 16 and 17 and N. van Baren, M. Marchand, B. Dreno, T. Dorval, S. Piperno, D. Lienard, B. Escudier, T. Boon, P. Coulie, M.-C. Bonnet, and P. Moingeon, manuscript in preparation). This vector will be referred to as ALVAC-MAGE. Vaccination included four injections of ALVAC-MAGE followed by three injections of peptides MAGE-1.A1 and MAGE-3.A1 without adjuvant. All vaccines were administered every 3 wk by intradermal and s.c. routes. Twenty-three HLA-A1 patients received the four ALVAC-MAGE injections, and five of them showed tumor regression.

Materials and Methods

Tetramer production

An HLA-A*0101 cDNA clone served as template to amplify the sequence coding for the extracellular domains (residues 1–276) of the HLA-A1 H chain with primers A1M8 (5′-AAGAAGGAGATATACCATGGGtTCaca cagtATGcgctAtTTttttacatccgtgtcccgg) and A27b (5′-ATGATGCAGGG ATCCTTATTATTCGAAGATGTCGTTCAGACCACCACCCAGCTCC CATCTCAGGGTG). A1M8 contains several base changes (small letters) designed to optimize protein expression in *Escherichia coli* BL21(DE3)pLysS. The PCR product was digested with *NcoI* and *SfiI* and cloned into a vector derived from pET3D (Stratagene, Heidelberg, Germany) and containing a BirA biotinylation site in frame with the 3′ end of the HLA sequence. Recombinant HLA molecules were folded in vitro with β_2-microglobulin (pHN1-β2m, kindly provided by P. Moss, Oxford University, Oxford, U.K.) and peptides EADPTGHSY (MAGE-1.A1), EVDPIGHLY (MAGE-3.A1), or VSDGGPNLY (influenza basic polymerase 1 (18)), as described previously (19). Soluble complexes purified by gel filtration were biotinylated using the BirA enzyme (Avidity LCC, Denver, CO). PE or allophycocyanin-labeled tetramers were produced by mixing the biotinylated complexes with Extravidin-PE (Sigma-Aldrich, St. Louis, MO) or streptavidin-allophycocyanin (Molecular Probes, Eugene, OR). Each tetramer batch was validated by staining CTL clones with the appropriate specificity, titered, and then used at the optimum concentration, usually 5 nM HLA H chain.

MLPC

PBMC isolated by Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation were cryopreserved in Iscove's medium supplemented with 10% human serum (HS) and 10% DMSO. After thawing, PBMC were resuspended at 10^7 cells/ml in Iscove's medium supplemented with 1% HS and divided into two groups. One group was incubated for 60 min at room temperature with peptide MAGE-1.A1 (20 μM). The other was incubated with MAGE-3.A1. These pulsed PBMC were washed, pooled, and distributed at 2.5×10^5 cells/0.2 ml in round-bottom microwells in Iscove's medium with HS (10%), L-arginine (116 mg/L), L-asparagine (36 mg/L), L-glutamine (216 mg/L), IL-2 (20 U/ml), IL-4 (10 ng/ml), and IL-7 (10 ng/ml). On day 7, 50% of the medium was replaced by fresh medium containing IL-2, IL-4, and IL-7. Peptide MAGE-3.A1 (20 μM) was added to all cultures. Peptide MAGE-1.A1 was added to all cultures 1 day later. During the second week of stimulation, the cultures were divided according to proliferation in medium containing IL-2 alone. Tetramer labeling was performed on day 14.

Improved protocol for the detection of tetramer-positive cells

We have observed in many MLPC/tetramer experiments that the proportions of cells labeled by a tetramer were <0.03% of the CD8 cells. As the background labeling by a tetramer is in the same range, we use two criteria to distinguish the cells that are specifically labeled by a tetramer from those that are labeled nonspecifically or that appear to be labeled because they display autofluorescence (see Fig. 1). We gate out the cells that display autofluorescence and those that are equally labeled by the relevant A1/MAGE tetramer and by a control HLA-A1 tetramer containing an influenza peptide. Briefly, MLPC were washed, resuspended in PBS with 1% HS, and incubated for 30 min at 37°C with the two HLA-A1 tetramers. Anti-CD8 Abs coupled to FITC (SK1; BD Biosciences, Mountain View, CA) were then added and after a further incubation for 30 min at 37°C, the cells were washed, fixed with 0.5% formaldehyde, and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Typical results are shown in Fig. 1 for four MLPC set up with PBMC from patient EB81 (*A–C*) or LB2196 (*D*) stimulated with peptide MAGE-3.A1. The classical CD8/tetramer plots are shown in column 2 for lymphocytes gated on their light scattering properties, as shown in column 1. Column 3 shows that the specificity of detection is improved by gating out cells that emit a higher than background fluorescence at 670 nm when excited at 488 nm. Considering that no dye emitting at 670 nm is present during labeling, the positive events in this "empty channel" are probably cells with a high autofluorescence. This autofluorescence is also apparent in the PE detection channel, leading to false positives. The usefulness of gating out these events is clearly apparent for MLPC D, which contains cells that appear to be $CD8^+$ and tetramer positive. With the autofluorescence correction, this culture can safely be considered negative. Column 4 shows the results when the labeling with the control tetramer is taken into account. In MLPC A and B, there are clearly CD8 cells that are labeled with the two tetramers, artificially increasing the sizes of the A1/MAGE-3^+ clusters detected with a single tetramer.

Typical labeling data of a MLPC/tetramer experiment are shown in Fig. 2.

T cell clones and TCR analysis

To derive stable CTL clones from the populations of tetramer-positive cells, which are usually clonal populations, cells stained by tetramer were seeded at one cell per well in round-bottom microplates using flow cytometry and stimulated by the addition of irradiated (100 Gy) allogenic PBMC (8×10^4/well) as feeder cells and irradiated allogenic HLA-A1 EBV-B cells (2×10^4/well) incubated with the MAGE-3.A1 peptide (20 μM) and washed in culture medium with IL-2 (100 U/ml), IL-4 (10 ng/ml), and IL-7 (10 ng/ml). The CTL clones were restimulated weekly by the addition of feeder cells and peptide-pulsed EBV-B cells in medium with growth factors. After ~3 wk, they were transferred into 2-ml wells and maintained by weekly restimulations with allogenic LG2-EBV-B cells and peptide-pulsed HLA-A1 tumor cells.

Total RNA from PBMC or tumor material was extracted with the Tripure reagent (Roche Diagnostics, Brussels, Belgium). Reverse transcription was performed at 42°C for 90 min with 200 U of Moloney murine leukemia virus reverse transcriptase mixed with 4 μl of 5× First Strand Buffer (Life Technologies, Merelbeke, Belgium), 2 μl of 20 μM oligo$(dT)_{15}$, 20 U of RNasin (Promega, Madison, WI), 2 μl of 100 mM DTT (Life Technologies), 1 μl of each dNTP at 10 mM each (Takara, Shiga, Japan), and diethyl pyrocarbonate-treated water in a total volume of 20 μl. cDNA served as template for PCR amplifications using panels of Vα- or Vβ-specific upstream primers and one downstream Cα or Cβ primer chosen on the basis of described panels of TCR V region oligonucleotides (20) and TCR sequences available at the International Immunogenetics Database of M.-P. Lefranc (http://imgt.cines.fr). PCR products were purified and sequenced to obtain a complete identification of the CDR3 region. The anti-MAGE-3.A1 CTL clones expressed the following rearranged TCR genes: CTL 35 of patient EB81: Vα12-Jα41 and Vβ24-Jβ1.2; CTL 103 of patient EB81: Vα21-Jα28 and Vβ5-Jβ2.7; CTL 1 of patient LAU147: Vα12-Jα43, Vβ24.1-Jβ2.7; and CTL 2 of patient NAP33: Vα29-Jα49 and Vβ15-Jβ1.2.

Clonotypic PCR

Frequency estimations using clonotypic PCR were based on limiting dilution analysis: PBMC were aliquoted in groups of 10^5–10^6 cells, and clonotypic RT-PCR was applied to all groups for the TCR α- and β-chains. The proportions of CD8 cells in the PBMC and of positive groups for the clonotypic PCR were used to calculate a frequency for a given CTL clone. Here follows the procedure for the anti-MAGE-3.A1 CTL clone of patient EB81. It involves three nested PCR amplifications. The rearranged TCR sequences were Vα12–1/Jα41 (5′-..ggtgaacGAGAAaaattcc..) and Vβ24/Jβ1–2 (5′-..cagtgatGCTGGGACAGCAATAtatggct..), with nucleotides that are not templated by V or J genes in capital letters and in lowercase letters the 3′ and 5′ ends of the V and J sequences, respectively. RNA was extracted from groups of PBMC using Tripure and converted to cDNA using the SuperScript II reverse transcriptase (Life Technologies) and an anchored oligo(dt)$_{21}$ primer, which contains a T7 promoter sequence at its 5′ end. All of the cDNA was used as template in a PCR amplification with forward primers corresponding to Vβ24 (LUR353: 5′-cacaaagacaggaaa gaggattatg) and Vα12 (LUR351: 5′-ttccagagggagccactg), and a reverse primer (5′-gccagtgaattgtaatacgactcac) matching the T7 sequence (for 94°C, 5 min (94°C, 45 s; 62°C, 60 s; 72°C, 120 s) for 22 cycles, 72°C, 10 min). Aliquots (1/100) of this amplified product were used as templates in nested PCR amplifications. For the TCRβ chain, a first amplification used LUR353 and a primer straddling the Jβ1–2/Cβ junction and a second amplification used another Vβ24 primer, downstream to LUR353, and a primer matching the CDR3β sequence. For the TCRα chain, a first amplification with LUR351 and a Cα primer and a second amplification with a CDR3α primer and a primer straddling the Jα41/Cα junction. The CDR3 primers did not completely cover the CDR3 regions, allowing us to check for the presence of the appropriate N nucleotides by sequencing the final PCR product.

Results

To analyze the anti-MAGE-1.A1 and anti-MAGE-3.A1 CTL responses of four patients who showed tumor regression after ALVAC-MAGE vaccination, we used in vitro restimulation of PBMC with the antigenic peptides over 2 wk, followed by labeling with A1/MAGE tetramers (15). To evaluate precursor frequencies, these MLPC were conducted under limiting dilution conditions. The logic of the selection of tetramer-labeled cells is shown in Fig. 1. A typical result is shown in Fig. 2. It should be noted that, as a result of the limiting dilution, the tetramer-labeled clusters in the positive microcultures (<10% of the microcultures) each represent a single clone. The tetramer-positive CD8 T cells were nevertheless cloned to eliminate contaminating lymphocytes. The stable CTL clones that were obtained were checked for their ability to lyse targets expressing Ag MAGE-3.A1. Only those microcultures that produced lytic clones were taken into account for the frequency evaluation. The TCR diversity was analyzed by sequencing. With this MLPC/tetramer/cloning approach, we observed a CTL response against Ag MAGE-3.A1 in three of the four patients. No CTL response directed against Ag MAGE-1.A1 was observed.

Regressor patient EB81

This patient had ~70 cutaneous metastases when she received the ALVAC-MAGE vaccine. About one-third of the metastases started to regress during these vaccinations, while the others regressed later during vaccination with the MAGE peptides (Fig. 3). All metastases eventually became undetectable, except for a new enlarged inguinal lymph node that was resected and found to contain tumor cells expressing *MAGE-1* and *MAGE-3*. Subsequently, the other nodes of the same region were removed, but none of them contained melanoma cells. Three years after the onset of vaccination, the patient was still free of detectable melanoma.

In prevaccination PBMC, no anti-MAGE-3.A1 T cells could be detected among 10^7 CD8 lymphocytes. After four injections of ALVAC-MAGE, the frequency of anti-MAGE-3.A1 CTLp rose to 3.7×10^{-6} of the CD8 cells, and it stayed between 10^{-6} and 10^{-5} during the vaccination with peptides (Fig. 3). We identified the TCR expressed by the anti-MAGE-3.A1 CTL found in 117 independent microcultures: 111 expressed the same TCR, which was

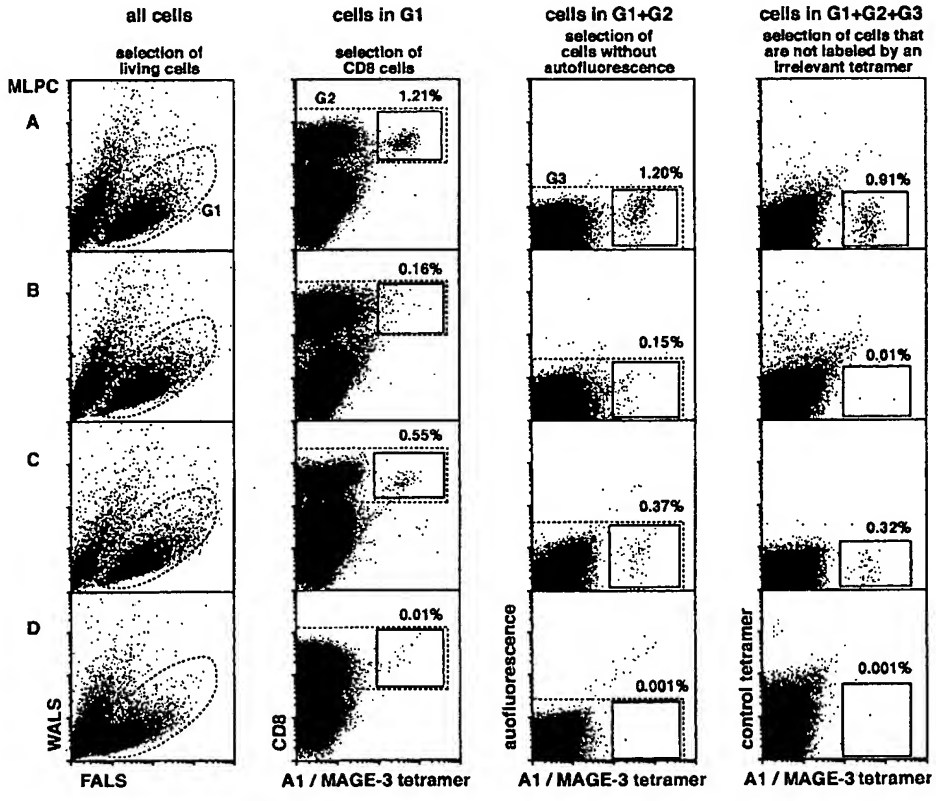

FIGURE 1. Procedure for the analysis of lymphocytes labeled by anti-CD8 Abs and A1/MAGE-3 tetramer. The indicated proportions (percent) correspond to CD8 cells that are labeled with the A1/MAGE-3 tetramer and satisfy the selection criteria mentioned above the columns. Results are shown for four independent anti-MAGE-3.A1 MLPC set up with PBMC from patient EB81 (*A–C*) or LB2196 (*D*) as described in *Materials and Methods*. After 14 days of restimulation with the antigenic peptide, cells were incubated with an A1/MAGE-3 tetramer labeled with PE, a control A1/influenza tetramer labeled with allophycocyanin, and anti-CD8 Abs labeled with fluorescein. In *column* 3, autofluorescence refers to fluorescence at 670 nm. *Columns 1–3* show the definition of three gates, indicated with dotted lines, and *columns 2–4* show the influences of these gates on the identification of clusters of cells labeled with the A1/MAGE-3 tetramer, indicated with squares. WALS, Wide angle light scatter; FALS, forward angle light scatter.

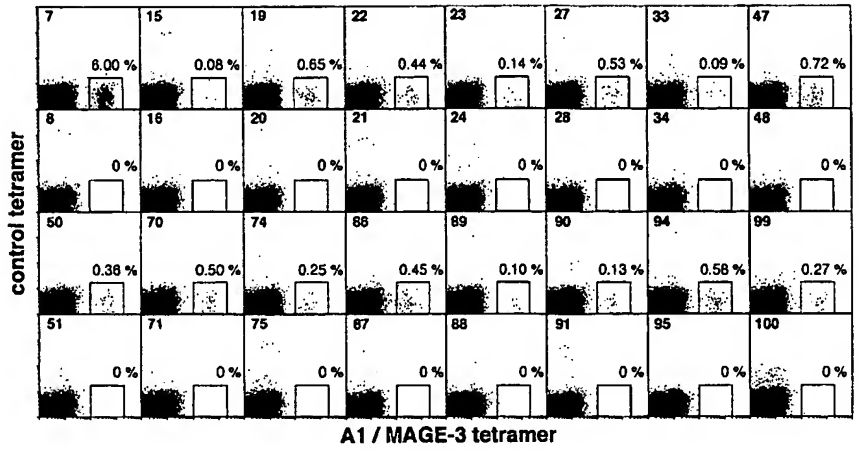

FIGURE 2. Estimation of the frequency of anti-MAGE-3.A1 CTLp in PBMC collected from patient EB81 after vaccination. One hundred one cultures were set up with 275,000 post 1 PBMC (Fig. 3) stimulated with the MAGE-1.A1 and MAGE-3.A1 peptides and IL-2, -4, and -7. The lymphocytes were restimulated on day 7 with peptides and cytokines and labeled on day 14 with anti-CD8 Abs, a HLA-A1 tetramer containing the MAGE-3.A1 peptide, and a control HLA-A1 tetramer containing an influenza peptide. The plots show only the $CD8^+$ lymphocytes, corresponding to 30–40% of the cells, of the 16 positive cultures in this experiment (*first* and *third rows*) and of 16 adjacent negative cultures (*second* and *fourth rows*). The proportions of $CD8^+$ lymphocytes that are specifically labeled with the A1/MAGE-3 tetramer are indicated. Considering that the PBMC contained 17% CD8 lymphocytes, these results indicate that the frequency of anti-MAGE-3.A1 T cells is 3.7×10^{-6} of the CD8 in this blood sample.

named TCR 35. Three CTL expressed another receptor, TCR 103, and three expressed unidentified TCR, but different from TCR 35. As shown in Fig. 4, CTL clones 35 and 103 lysed specifically not only autologous EBV-B cells pulsed with the MAGE-3.A1 peptide but also the autologous melanoma cells, which express *MAGE-3*.

PCR amplifications specific for the TCR 35 Vα and Vβ rearrangements were applied directly to cDNA obtained from groups of PBMC. The observed frequencies closely matched those found by the MLPC/tetramer approach (Fig. 3). We conclude that the blood frequency of anti-MAGE-3.A1 CTL increased from $<10^{-7}$ to 3×10^{-6} of the CD8 after ALVAC vaccination and that this response was essentially monoclonal.

Lymph nodes were analyzed to find out whether they contained anti-MAGE-3.A1 CTL other than those found in blood. In a MLPC/tetramer experiment conducted with cells of the metastatic lymph node resected in April 2000, we found CTL 35 and CTL 103 and no other anti-MAGE-3.A1 CTL. In the surrounding lymph nodes, which did not contain tumor cells, MLPC/tetramer experiments indicated an anti-MAGE-3.A1 CTLp frequency of 2×10^{-6} of the CD8, and clonotypic PCR indicated that all of these CTL expressed TCR 35.

We explored whether a set of vaccinations given at short intervals could boost the anti-MAGE-3.A1 CTL response. From February 2001, the patient received five weekly injections of peptide MAGE-3.A1. Anti-MAGE-3.A1 CTLp frequencies were 10^{-6} of the CD8 before the boost (Fig. 3, post 6) and 1.7×10^{-6} one week after the fifth peptide injection (post 7). Six months later, the patient received 4 weekly injections of ALVAC-MAGE. The anti-

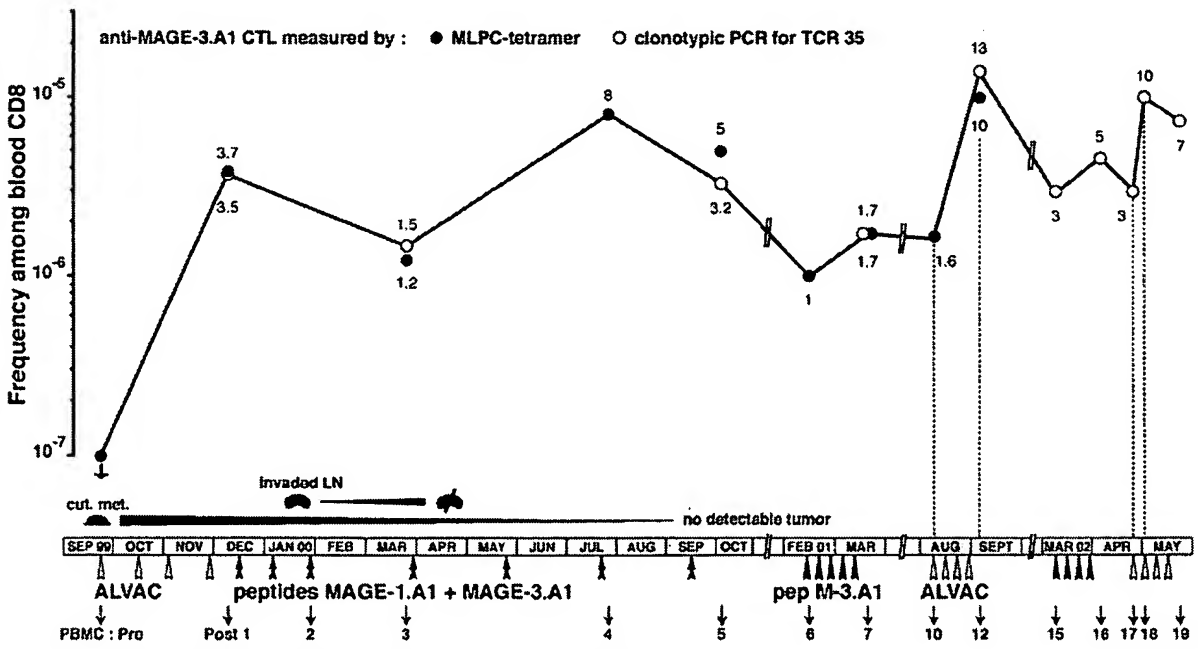

FIGURE 3. Clinical evolution of patient EB81 and frequencies of anti-MAGE-3.A1 CTL.

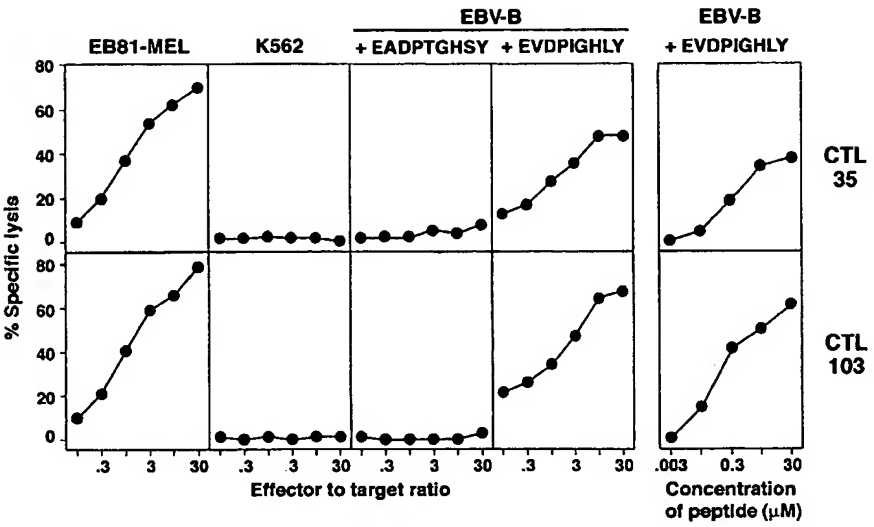

FIGURE 4. Lytic activity of anti-MAGE-3.A1 CTL clones 35 and 103 from patient EB81. ^{51}Cr-labeled targets included autologous melanoma cells EB81-MEL, NK target cells K562, and autologous EBV-transformed B cells incubated with peptides MAGE-1.A1 (EADPTGHSY) or MAGE-3.A1 (EVDPIGHLY) at 2.5 μM. For peptide titration (*right panels*), CTL clones were added at an E:T cell ratio of 10.

MAGE-3.A1 CTLp frequency increased almost 10-fold to 10^{-5} of the CD8 one week after the fourth injection (post 12). Clonotypic PCR for TCR 35 indicated a frequency of 1.3×10^{-5} in this blood sample, confirming the increased CTL frequency and indicating that it corresponded to CTL 35 and not to a new CTL. New series of peptide and virus injections were administered in 2002. Here again, the blood frequency of CTL 35 did not increase significantly after the injections of peptide, but increased 3-fold after a single injection of ALVAC-MAGE (Fig. 3). It did not increase further after three additional ALVAC injections.

Regressor patient LAU147

This patient had one subcutaneous metastasis at study entry. It regressed completely after the fourth ALVAC-MAGE injection. But the detection of a brain metastasis resulted in patient withdrawal from the study before the injections of peptides. Before

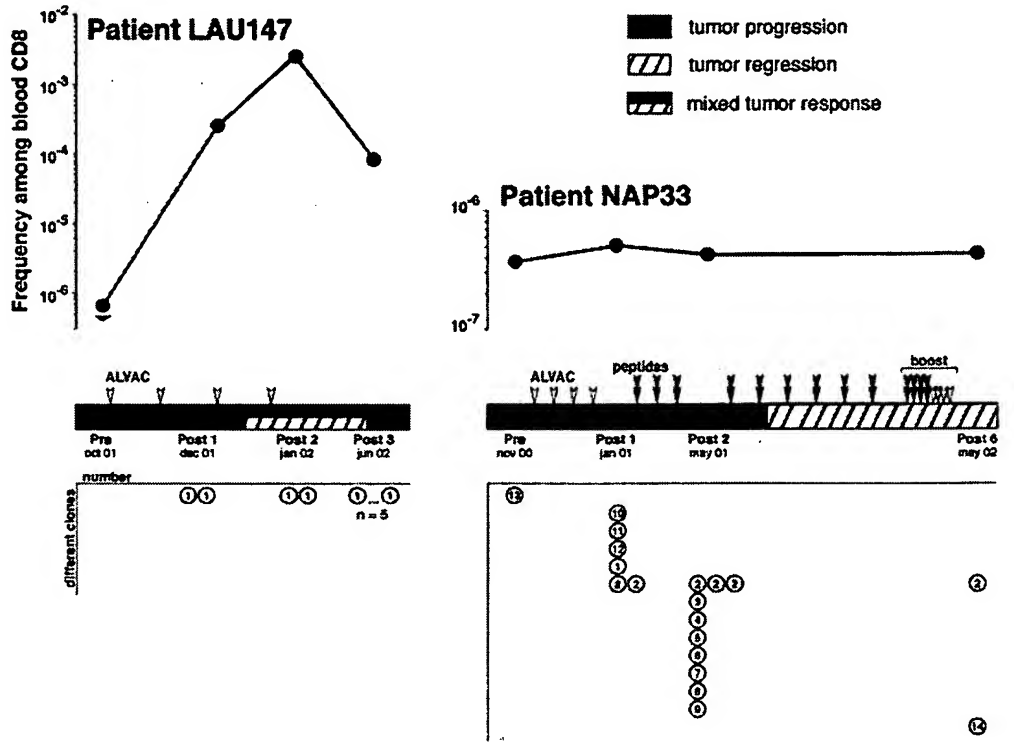

FIGURE 5. Frequency and diversity of anti-MAGE-3.A1 CTL. The *top panels* indicate the frequencies of anti-MAGE-3.A1 CTLp measured with tetramers after in vitro peptide restimulation in limiting dilution conditions. The clinical evolution of the patients is summarized on the *x*-axis, with vaccinations and blood collections. For patient NAP33, the boost included 4 weekly injections of peptides MAGE-1.A1 and MAGE-3.A1, followed 1 wk later by 4 weekly injections of ALVAC-MAGE. Blood was collected 1 wk after the last ALVAC injection. The *bottom panels* represent the CTL clones that were derived from cells stained with tetramer. Each circle represents one clone derived from one population of 250,000 PBMC stimulated with peptide. Each number represents a different TCR sequence. Numbering starts at 1 for each patient. Similar numbers assigned to CTL of different patients do not represent TCR similarities. When several independent CTL clones share the same TCR sequence, they are aligned horizontally.

Table I. *Analysis of patients without tumor regression*

Patient	Anti-MAGE-3.A1 CTLp Frequency[a] Pre	Post	CTL Response
NAP36	$<1.4 \times 10^{-7}$	2.5×10^{-6}	+
VUB39	1.1×10^{-6}	9.3×10^{-6}	+
BB132	nt[b]	1.1×10^{-7}	−
LAU622	nt	$<2.7 \times 10^{-7}$	−
LB2196	$<3.5 \times 10^{-7}$	$<3.5 \times 10^{-7}$	−
LAU407	nt	$<3.5 \times 10^{-7}$	−
CP69	nt	$<4.7 \times 10^{-7}$	−
KUL73	nt	$<5.0 \times 10^{-7}$	−
NAP35	nt	$<5.2 \times 10^{-7}$	−
UZG10	nt	$<9.5 \times 10^{-7}$	−
IGR37	nt	$<9.6 \times 10^{-7}$	−
LB2291	nt	$<1.0 \times 10^{-6}$	−

[a] Among blood CD8 lymphocytes.
[b] nt, Not tested.

vaccination, the blood frequency of anti-MAGE-3.A1 CTLp was 4×10^{-7} of the CD8 cells (Fig. 5). At the time of the third ALVAC injection, this frequency was 2.5×10^{-4} of the CD8, and it increased up to 3×10^{-3} after the fourth injection. Five months later, it had decreased to 8×10^{-5} of the CD8. Nine anti-MAGE-3.A1 CTL clones were derived. All expressed the same TCR.

Regressor patient NAP33

This patient had three subcutaneous metastases at the onset of vaccination. These tumors progressed and a fourth metastasis appeared. Approximately 6 mo after the first vaccination, all metastases regressed slowly until complete clinical disappearance. Before vaccination, the frequency of anti-MAGE-3.A1 CTLp was 3.8×10^{-7} of blood CD8 cells and it did not increase after vaccination (Fig. 5). However, TCR analysis of 18 anti-MAGE-3.A1 CTL clones obtained after vaccination indicated that one clonotype, TCR 2, was repeated 6 times, whereas the 11 others were different. We conclude that CTL 2 expanded following vaccination.

Patient NAP33 also received series of weekly booster injections of peptides and ALVAC-MAGE (Fig. 5). The frequency of anti-MAGE-3.A1 CTLp did not increase after these vaccinations.

Regressor patient CP67

This patient showed regression of subcutaneous and lymph node metastases after the vaccinations with ALVAC-MAGE. No anti-MAGE-3.A1 or anti-MAGE-1.A1 CTLp could be found among 18×10^6 postvaccination PBMC, indicating frequencies below 3.7×10^{-7} of CD8 cells.

Patients without tumor regression

Twelve of 16 HLA-A1-vaccinated patients who did not display tumor regression were analyzed (Table I). An anti-MAGE-3.A1 CTL response was observed in two patients. In patient NAP36, the pre- and postvaccination CTLp frequencies were $<1.4 \times 10^{-7}$ and 2.5×10^{-6} of the CD8, respectively. Six of seven postvaccination CTL clones shared the same TCR, indicating that this CTL expanded after vaccination. For patient VUB39, pre- and postvaccination frequencies were 1.1×10^{-6} and 9.3×10^{-6} of the CD8. One CTL clone was already repeated before vaccination, suggesting that this patient had mounted a spontaneous anti-MAGE-3.A1 CTL response.

No evidence for an anti-MAGE-3.A1 CTL response could be found in the other patients. For nine patients, no anti-MAGE-3.A1 T cells could be detected in MLPC/tetramer experiments, resulting in frequency estimations below $3–9 \times 10^{-7}$ of the CD8. For patient BB132, one microculture contained tetramer-positive cells, resulting in a frequency of 10^{-7} of the CD8.

Discussion

Our results demonstrate that vaccination with ALVAC-MAGE induced a CTL response in patients EB81 and LAU147, as a CTL response was observed before the peptide boost. This is the first demonstration that recombinant ALVAC can induce CTL responses against a tumor-specific Ag in cancer patients with a detectable tumor bearing this Ag. We also observed that booster injections of ALVAC-MAGE increased the blood frequency of previously activated CTL clones (see posts 12 and 18 in patient EB81 and post 2 in patient LAU147). Considering that in mice vaccinated with ALVAC encoding the P1A Ag of mastocytoma P815 (our unpublished observations) and in humans receiving the smallpox vaccine (21) the intensity of the T cell responses correlated with the dose of virus, it will be of great interest to examine the efficacy of a higher dose of ALVAC-MAGE.

The CTL responses that we have observed appear stable: when a responding CTL clone was detected, its blood frequency always remained detectable. But the intensities of the observed anti-MAGE-3.A1 CTL responses varied widely from one patient to another. In patients EB81, LAU147, and NAP33, the blood frequencies of the amplified CTL clone were 3×10^{-6}, 3×10^{-3}, and 10^{-7} of the CD8 cells, respectively. It is worth noting that with a total number of $\sim 4 \times 10^{10}$ CD8 lymphocytes in a human individual, a frequency of 10^{-7} corresponds to a population of 4000 cells or 12 divisions after the activation of a naive precursor. Assuming that antivaccine T cells initiate the tumor regression process observed after peptide or ALVAC-MAGE vaccinations and that the CTL clones that we detected represent all of these antivaccine T cells, our results indicate that the initiation of the tumor regressions did not require a large number of antivaccine T cells.

The CTL responses observed after ALVAC-MAGE vaccination were monoclonal for patients LAU147 and NAP33. In patient EB81, one clone represented at least 95% of the response. Incidentally, the TCR sequences of the CTL found in all of these patients were completely different. Monoclonality has also been observed after vaccination with peptide MAGE-3.A1 without adjuvant (13, 15). It is, of course, impossible to exclude that other anti-MAGE-3.A1 T cells were activated in vivo, but failed to proliferate in vitro under our restimulation conditions. However, it is worth noting that when we applied the same MLPC/tetramer/cloning approach to metastatic melanoma patients receiving mature dendritic cells pulsed with peptide MAGE-3.A1, we observed polyclonal CTL responses (22). Presumably, the capacity of a peptide or ALVAC-MAGE injection to activate specific T cells is very limited in time and space, leading to at best a single hit. Improved vaccination modalities are expected to activate several T cell clones, and this is indeed the case in patients vaccinated with peptide-pulsed dendritic cells. In patients vaccinated with peptide or ALVAC-MAGE, the monoclonality of the response provides the opportunity to track down all of the antivaccine CTL directly in the blood using clonotypic PCR, to analyze their phenotype or function (15), and to assess their presence in regressing and nonregressing tumors.

In this study, we have analyzed four patients who showed tumor regression and we have observed three CTL responses. Assuming that a CTL response nevertheless occurred in the other patient, there are several plausible explanations for our inability to detect it. First, the patient could produce T cells against peptide MAGE-1.A1 or MAGE-3.A1 presented by a HLA molecule other than A1. Such T cells would escape detection by the tetramer method, which is locked on one presenting HLA molecule. In line with this

possibility is our observation that peptides MAGE-1.A1 and MAGE-3.A1 can also be presented by HLA-B35 molecules (23, 24). However, we have actively explored alternative HLA-peptide combinations with peptides MAGE-1A.1 and MAGE-3.A1 in the regressor patient without detectable HLA-A1-restricted CTL, and we have failed to find a CTL response against a new combination. Another possibility is that a monoclonal CTL response escaped detection by the MLPC/tetramer approach due to the inability of these T cells to grow under our restimulation conditions. Finally, mice were shown to reject tumors of ~0.5 cm^3 with ~20,000 tumor-specific CTL. In humans, with 4×10^{10} CD8 T cells, this number of CTL corresponds to a blood frequency of 5×10^{-7} (15). It is therefore possible that a tumor regression process could be initiated by antivaccine CTL present in the blood at a frequency of about or below 10^{-7} of the CD8, rendering their detection almost impossible with present methods.

The evolution of the patients of the ALVAC-MAGE vaccination study mentioned in this report will be described in detail elsewhere (N. van Baren, M. Marchand, B. Dreno, T. Dorval, S. Piperno, D. Lienard, B. Escudier, T. Boon, P. Coulie, M.-C. Bonnet, and P. Moingeon, manuscript in preparation). Thirty melanoma patients received at least four ALVAC-MAGE vaccinations and were considered evaluable for tumor response. Of these, 21 were HLA-A1. The other patients expressed HLA-B35, which also presents the MAGE-1.A1 and MAGE-3.A1 peptides (23, 24). Tumor regressions were observed in 5 of the 21 HLA-A1 patients and CTL responses in 3 of the 4 who were tested. For the 16 HLA-A1 progressor patients. CTL responses were observed in 2 of the 12 that were tested. Adding these results to those obtained in peptide vaccination studies ((9, 13) and our unpublished results), regressions of melanoma metastases were observed in 18 of 91 patients and anti-MAGE-3.A1 CTL responses were detected in 5 of 10 regressor patients and 2 of 18 progressors. These numbers suggest that there is a correlation between the occurrence of these CTL responses and the tumor regressions, but this needs to be confirmed with larger numbers.

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ORIGINAL REPORT

Tumoral and Immunologic Response After Vaccination of Melanoma Patients With an ALVAC Virus Encoding MAGE Antigens Recognized by T Cells

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ABSTRACT

Purpose

To evaluate the toxicity, antitumoral effectiveness, and immunogenicity of repeated vaccinations with ALVAC miniMAGE-1/3, a recombinant canarypox virus containing a minigene encoding antigenic peptides MAGE-$3_{168-176}$ and MAGE-$1_{161-169}$, which are presented by HLA-A1 and B35 on tumor cells and can be recognized by cytolytic T lymphocytes (CTLs).

Materials and Methods

The vaccination schedule comprised four sequential injections of the recombinant virus, followed by three booster vaccinations with the MAGE-$3_{168-176}$ and MAGE-$1_{161-169}$ peptides. The vaccines were administered, both intradermally and subcutaneously, at 3-week intervals.

Results

Forty patients with advanced cancer were treated, including 37 melanoma patients. The vaccines were generally well tolerated with moderate adverse events, consisting mainly of transient inflammatory reactions at the virus injection sites. Among the 30 melanoma patients assessable for tumor response, a partial response was observed in one patient, and disease stabilization in two others. The remaining patients had progressive disease. Among the patients with stable or progressive disease, five showed evidence of tumor regression. A CTL response against the MAGE-3 vaccine antigen was detected in three of four patients with tumor regression, and in only one of 11 patients without regression.

Conclusion

Repeated vaccination with ALVAC miniMAGE-1/3 is associated with tumor regression and with a detectable CTL response in a minority of melanoma patients. There is a significant correlation between tumor regression and CTL response. The contribution of vaccine-induced CTL in the tumor regression process is discussed in view of the immunologic events that could be analyzed in detail in one patient.

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INTRODUCTION

Tumor cells carry antigenic peptides bound to HLA class I molecules that can be recognized by autologous cytolytic T lymphocytes (CTLs). Some of these antigens are absent from normal tissues, and thus constitute safe targets for T cell-mediated immunotherapy of cancer.[1] An important category of tumor specific antigens include those encoded by cancer-germline genes such as members of the *MAGE*, *BAGE*, *GAGE* and *LAGE-1/NY-ESO-1* gene families. These antigens are expressed by many melanomas, transitional bladder cancers, head and neck squamous cell carcinomas, non–small-cell lung cancers, esophageal cancers, and multiple myelomas.[2]

Tumor vaccine candidates containing the MAGE-3.A1 antigen, nonapeptide MAGE-$3_{168-176}$ presented by HLA-A1, have been investigated in small-scale clinical trials. In a pilot study, the synthetic MAGE-3.A1 peptide was administered to 45 HLA-A1 patients with *MAGE-3* expressing melanoma, by subcutaneous (SC) and intradermal (ID) injections of 100 or 300 μg of peptide on three occasions at monthly intervals. No significant toxicity was reported. Of the 25 melanoma patients with measurable disease who received all three immunizations, seven displayed tumor regressions. We observed three complete responses, one partial response, and three mixed responses.[3] In a phase I/II trial, the recombinant MAGE-3 protein was tested as a vaccine formulation in patients with *MAGE-3* expressing cancer, mainly melanoma. The patients received either 30, 100, or 300 μg of the protein, with or without the immunological adjuvants MPL and QS21, repeatedly by intramuscular injection. No severe toxicity was reported. Among 33 assessable melanoma patients, four experienced regressions of metastatic lesions, two partial and two mixed responses. A partial response was also observed in a patient with metastatic bladder cancer.[4] The same MAGE-3 protein was administered ID and SC without immunologic adjuvant to 26 patients with metastatic, nonvisceral melanoma. Five regressions, one partial response and four mixed responses, were reported (W.H.J. Kruit et al, unpublished observations). In another clinical study, patients with advanced metastatic melanoma were vaccinated with autologous dendritic cells pulsed with the MAGE-3.A1 peptide administered subcutaneously and intravenously. Six of eleven patients immunized with this vaccine showed tumor regressions, all being mixed responses.[5]

In our initial vaccination study with the MAGE-3.A1 peptide, we did not observe anti-vaccine CTL responses even in those patients who showed tumor regressions. This indicated the absence of strong CTL responses (ie, responses involving CTL frequency above 10^{-4} of CD8 T cells, our detection threshold at that time). More recently, a new approach with an improved sensitivity of approximately 8×10^{-7}, involving lymphocyte-peptide culture and the use of HLA/peptide tetramers, was used to document a significant increase in cytolytic T lymphocyte precursor (CTLp) frequency in a patient who showed tumor regression following vaccination with the MAGE-3.A1 peptide. This method also showed that the CTL response was monoclonal.[6] It was extended to 19 other patients who received this peptide without adjuvant. None had a detectable CTL response, indicating that this vaccine is weakly immunogenic.[7] In patients vaccinated with the MAGE-3 protein, sensitive monitoring of T lymphocyte responses with HLA/peptide tetramers was not possible because of the great diversity of antigenic peptides that a protein vaccine can generate.

Among other potential vaccine candidates aimed at inducing CTL responses to tumor antigens, viral vector–based approaches offer potential advantages. More specifically, viral antigens are often very immunogenic, as indicated by the strong cellular immune responses that can be observed during human viral diseases. One of the possible reasons for this strong immunogenicity is the fact that viruses are potent activators of innate immunity, which in turn can boost specific immune responses. Cells infected by viruses can express foreign genes that have been inserted into the viral genome. Like endogenous proteins, the foreign gene products can be processed into peptides that are displayed at the cell surface by HLA molecules, allowing primary cellular immune responses to such antigens to be induced by infecting professional antigen-presenting cells with recombinant viruses.[8] Among possible vector candidates, avian poxviruses deserve particular attention. They have the ability to infect a wide variety of cell types in various hosts, including mammals, but their replication is restricted to avian cells, which prevents them from causing viral disease in humans. ALVAC is an attenuated canarypox virus that has been extensively tested in animal models.[9] Its excellent safety profile and its capacity to induce immune responses in humans have been established in a series of clinical trials.[10-15]

We report here clinical observations made on 40 patients with advanced cancer who received vaccinations with ALVAC miniMAGE-1/3, a recombinant ALVAC virus that contains a minigene coding for a MAGE-3 and a MAGE-1 antigen. These priming immunizations were followed by booster vaccinations with the two corresponding peptides. We also provide a synthesis of the analysis of the CTL responses of the patients, carried out with a sensitive detection approach.

MATERIALS AND METHODS

CTL Recognition of Cells Infected With ALVAC miniMAGE-1/3 Virus

Dendritic cells were derived from monocytes isolated from the blood of an HLA-A1 hemochromatosis patient, as described previously.[16] They were distributed in microwells at 10,000 cells per well and were infected at increasing multiplicities of infection for 2 hours with either ALVAC miniMAGE-1/3 or a control ALVAC virus expressing β-galactosidase. Infected cells were then washed and incubated for 20 hours with 3,000 cells of either CTL clone 82/30 or CTL clone 20/38, which recognize the MAGE-1.A1 and MAGE-3.A1 antigens, respectively.[17,18] The concentration of interferon (IFN) -γ, which is released by activated CTL, was measured in the supernatant by enzyme-linked immunosorbent assay (ELISA).

Vaccine Production

ALVAC miniMAGE-1/3 clinical material was produced by Aventis Pasteur (Marcy l'Etoile, France). In brief, the candidate vaccine construct was derived as follows. A cDNA encoding a polypeptide including amino acids 149 to 181 of the MAGE-1 protein, followed by an NKRK protease cleavage site and by amino acids 168 to 176 of the MAGE-3 protein, was ligated into a donor plasmid downstream of a vaccinia H6 early/late

promoter element.[19] The recombinant plasmid, harboring this expression cassette, was transfected into primary chick embryo fibroblasts, which were then infected with wild-type ALVAC virus. After successive rounds of plaque purification and selection, a recombinant ALVAC virus containing the appropriate expression cassette inserted into the C6 nonessential site, was isolated and amplified. The recombinant virus was confirmed by DNA restriction analysis and by nucleotide sequence analysis and was designated vCP 1469A. It is further referred to as ALVAC miniMAGE-1/3. The clinical batch S3420 used in this trial was produced according to the good manufacturing practice (GMP) guidelines. The viral vaccine was formulated as a lyophilized powder corresponding to a viral dose of 1.23×10^7 $CCID_{50}$ (50% of the cell culture infectious dose). The vaccine vials were kept stored at 4°C, and were reconstituted before administration with 1 ml of water for injection.

Peptides MAGE-1.A1 (amino acid sequence EADPTGHSY) and MAGE-3.A1 (EVDPIGHLY) were synthesized by Multiple Peptide Systems (Sunnyvale, CA) and were provided by Aventis Pasteur in accordance with GMP (batches D01164 and D01165, respectively). They were formulated in solution in phosphate buffered saline, pH 7.4, at a concentration of 600 μg/ml. Vials were kept frozen at –80°C and were thawed just before injection.

Patient Eligibility Criteria

Patients enrolled on the trial were required to have measurable advanced malignancy of one of the following histologic types: cutaneous melanoma, non–small-cell lung cancer (NSCLC) or head and neck, esophageal, or bladder cancer. Other inclusion criteria were expression of HLA-A1 or HLA-B35, the two HLA types that are known to present the MAGE-$1_{161\text{-}169}$ and MAGE-$3_{168\text{-}176}$ epitopes, expression of genes *MAGE-1* or *MAGE-3* by the tumor as determined by reverse transcriptase polymerase chain reaction on a frozen tumor biopsy, age 18 years or older, and WHO–Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1. Patients with abnormal organ function, brain metastasis, a second neoplasm, any other severe disease, or a known allergy to egg products or to neomycin, used in the production of ALVAC miniMAGE-1/3, were excluded. No chemotherapy, radiotherapy, or immunotherapy was allowed during the month preceding the first vaccination. All patients provided written informed consent before inclusion.

Study Design

This study was designed as a multicentric prospective open-label phase I/II trial to investigate the safety and toxicity (primary objective), and the antitumoral activity and immunogenicity (secondary objectives) of ALVAC miniMAGE-1/3 in patients with advanced cancer, with a focus on melanoma. The vaccination schedule started with 4 priming vaccinations with ALVAC miniMAGE-1/3 at 3-week intervals. The fixed virus dose, which was determined by the titer of the available clinical batch, was 1.23×10^7 $CCID_{50}$. After reconstitution, the viral suspension was injected in two intradermal sites (0.1 ml each) and two subcutaneous sites (0.4 mL each), in the arms and the anterior aspect of the thighs. Unless the disease had progressed in such a way that the patient needed another treatment, the ALVAC vaccinations were followed after 3 weeks by three booster vaccinations with the MAGE-3.A1 and MAGE-1.A1 peptides at 3-week intervals. Each peptide was injected once intradermally (60 μg) and once subcutaneously (240 μg), also in the arms and thighs. No injections were administered in extremities in which an axillary or inguinal lymph node dissection had been performed. Tumor staging comprised clinical evaluation of skin lesions and computed tomography scans of brain, chest, and abdomen. They were performed within 1 month before vaccination, and were repeated 2 weeks after the fourth ALVAC vaccination, and when applicable 4 and 8 weeks after the third peptide vaccination. Peripheral blood mononuclear cell (PBMC) collections were done at study entry, two weeks after the fourth ALVAC vaccination, and when possible 8 weeks after the third peptide vaccination. PBMCs were obtained either by leukapheresis or by the isolation of the buffy-coat from 500 mL of centrifuged venous blood. Separated PBMCs were purified by Lymphoprep (Nycomed, Oslo, Norway) gradient followed by several washing steps, and were frozen at –80°C or in liquid nitrogen, in Iscove's medium containing 10% human serum and 10% dimethyl sulfoxide. Serum for the detection of vaccine induced antibodies was collected at the same timepoints as PBMC, and was kept frozen at −20°C. This trial was performed according to the good clinical practice guidelines. All research activities were approved by the relevant regulatory bodies and by the institutional review board (IRB) at each participating site before study initiation.

Patients with a favorable course of the disease were offered the possibility to receive additional vaccinations with the two peptides at decreasing frequency, on a compassionate basis. In addition, a few of these patients received booster vaccinations with peptides and ALVAC for the purpose of analyzing their impact on the anti-vaccine CTL response. These complementary research activities were compliant to national regulations and received prior IRB approval and written informed consent from the patients.

Clinical Evaluation of the Patients

Adverse events were graded according to the National Cancer Institute of Canada Clinical Trial Group (NCIC CTG) common toxicity criteria scale.[20] The relationship between each adverse event and the experimental treatment was evaluated as definitely, probably, possibly, or not related by the clinical investigators. Adverse events that were considered as definitely, probably, or possibly related to the treatment are reported here as adverse reactions.

Tumor response was defined according to the WHO criteria.[21,22] Evaluation took place 4 weeks after the seventh vaccination or at time of study removal when appropriate. Objective responses and disease stabilizations were confirmed at least 4 weeks thereafter. For cutaneous melanoma, mixed responses (ie, regression of some target lesions while others remain stable, progress, or appear simultaneously), although formally classified as stable or progressive disease in the WHO classification, were documented as well. A long-term follow-up of all included patients was realized at regular intervals until death.

Analysis of the Immune Response to the Vaccine

CTL responses to the MAGE-3.A1 and MAGE-1.A1 antigens were assessed in HLA-A1 patients by mixed lymphocyte-peptide culture (MLPC)/tetramer/cloning, as described previously.[6,23] This approach allows to measure the specific cytolytic T lymphocyte precursor frequency in the blood, after in vitro restimulation of PBMCs with either the MAGE-3.A1 or MAGE-1.A1 peptide under limiting dilution conditions, followed by staining of responder cells with the A*0101/MAGE-3 or A*0101/MAGE-1 tetramer, respectively, and by cloning and characterization of tetramer stained CTL. The same assay was performed in HLA-B*3501 patients, using the B*3501/MAGE-3 and the B*3 enzyme–linked

immunoSPOT (ELISPOT) as described previously.[24] For the present analysis, HLA class I–transfected K562 cells pulsed with the appropriate peptide were used to stimulate CD8+ cells isolated from pre- and postimmune PBMCs.[25]

Antibody responses to the ALVAC virus and to the MAGE-1 and MAGE-3 proteins were assessed in the serum by ELISA, as described previously.[26]

RESULTS

A pilot clinical study was performed to evaluate the effect of priming with an ALVAC-MAGE vaccine candidate in patients with advanced malignancy. The ALVAC-MAGE construct coded for two antigenic peptides, MAGE-$3_{168-176}$ and MAGE-$1_{161-169}$, which are recognized by T cells on both HLA-A1 and HLA-B35. The MAGE expression cassette is represented schematically in Figure 1. Expression of the MAGE epitopes in infected cells was verified by the fact that ALVAC miniMAGE-1/3 virus rendered infected dendritic cells from an HLA-A1 individual capable of presenting the HLA-A1-restricted MAGE-$3_{168-176}$ and MAGE-$1_{161-169}$ epitopes to specific CTL clones (Fig 2). Similar results were obtained with HLA-B35 dendritic cells.[27,28]

The complete immunization of patients involved four vaccinations with this ALVAC miniMAGE-1/3 virus, followed by three boosting vaccinations with the synthetic peptides MAGE-$3_{168-176}$ and MAGE-$1_{161-169}$ without adjuvant, all administered intradermally and subcutaneously at 3-week intervals (Fig 3). These routes of administration were chosen because the skin contains a high density of antigen-presenting cells and lymphatic vessels. The choice of four ALVAC administrations was based on previous experience with an ALVAC-HIV construct, which showed that the rate of anti-HIV CTL responses increased further after the third and fourth vaccination, but remained stable after the fifth.[29]

Patient Characteristics

From September 1999 to December 2001, 40 patients were enrolled on the study. They comprised 18 men and 22 women with age ranging from 28 to 86 years, and with a mean age of 54 years. Thirty-seven patients had stage III or IV cutaneous melanoma, two had stage IV NSCLC and one had stage IV head and neck carcinoma. The main features of the patients are displayed in Table 1. Nine patients were removed from the study before they received the fourth ALVAC vaccination, due to the early death of two individuals, and to rapidly progressing disease for the others. Among the 31 patients who received four ALVAC vaccinations, 12 also completed the three peptide vaccinations.

Toxicity

The 40 patients enrolled in the trial received at least one vaccination with ALVAC and were evaluated for safety and toxicity after each vaccination according to the NCIC CTG scale. A total of 146 vaccinations with ALVAC and 46 vaccinations with the two peptides were performed. They were generally well tolerated. No patient was removed from the trial as a result of toxicity. No adverse reactions above grade 3 were reported. The frequency or severity of adverse reactions did not increase with the number of injections.

Adverse reactions at the ALVAC injection sites occurred very frequently. They were immediate and usually mild to moderate in intensity. They consisted mainly of extended redness, but edema, induration, and pain were also observed. No instance of skin necrosis was observed. There were no major differences between reactions at intradermal and subcutaneous injection sites, except that intradermal reactions usually appeared earlier, lasted for longer and were slightly less severe. Local inflammation was already noticeable after the first ALVAC injection, and did not vary strongly with subsequent administrations. These observations, coupled to the appearance of occasional flu-like symptoms and a frequent increase in plasma levels of C-reactive protein (data not shown), are consistent with the activation of innate immunity mechanisms. Local adverse reactions at peptide injection sites were rare and mild. All local reactions resolved within days.

Grade 1 and 2 systemic reactions reported after ALVAC vaccination occurred frequently, whereas grade 3 reactions were rare (Table 1). These systemic reactions consisted of asthenia (reported after 33% of ALVAC injections), grade 2 or 3 fever (22%), headache (19%), myalgia (16%), arthralgia (14%), nausea (10%), and pain (6%). Systemic reactions after peptide vaccinations were less frequent and less severe. They consisted of asthenia (after 15% of peptide injections), grade 2 fever (15%), pain (10%), headache (7%), myalgia (7%), and arthralgia (7%). This apparent difference between the ALVAC and peptides vaccines might be biased by the early removal of patients with the poorest clinical condition, who presumably have a higher probability of experiencing adverse events.

Nineteen serious adverse events (SAEs) were reported in 18 patients (Table 1). Fifteen of these SAEs were

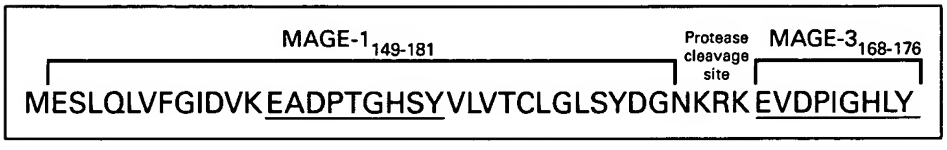

Fig 1. Amino acid sequence of the polypeptide encoded by the minigene inserted in the ALVAC viral genome. The MAGE-$1_{161-169}$ and MAGE-$3_{168-176}$ antigenic peptides, both presented by HLA-A1 and HLA-B35, are underlined.

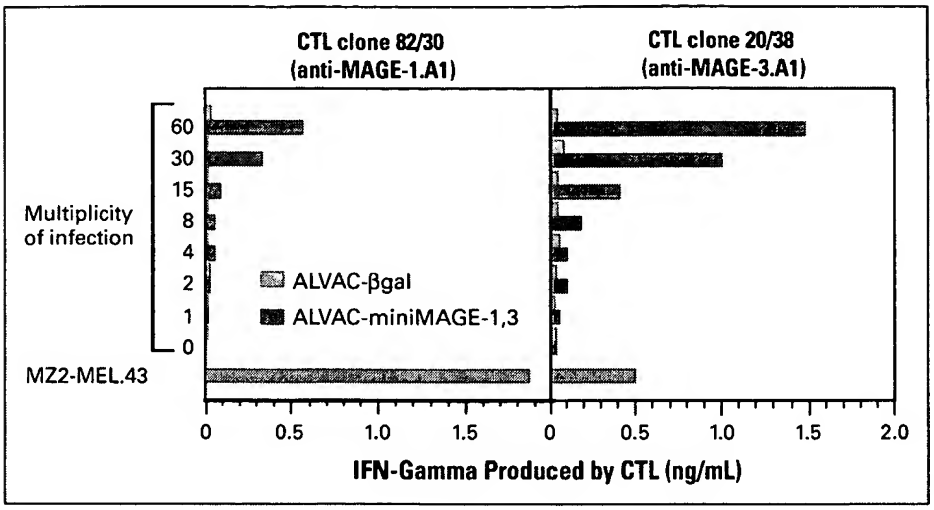

Fig 2. Immunogenicity of the viral construct assessed in vitro. HLA-A1 dendritic cells (DCs) were infected with ALVAC miniMAGE-1/3, or ALVAC-βgal as control. DC were distributed in microwells, infected at increasing multiplicities of infection for 2 hours, and washed. The HLA-A1, *MAGE-1* and *MAGE-3* expressing melanoma cell line MZ2-MEL was included as positive control. Anti-MAGE-1.A1 cytolytic T lymphocyte (CTL) 82/30 or anti-MAGE-3.A1 CTL 20/38 were added to the target cells. After 20 hours, interferon gamma produced by each CTL clone was measured by enzyme-linked immunosorbent assay (ELISA) in the supernatant.

considered clearly as unrelated to the study drugs. The four remaining SAEs were considered as possibly or probably related to the ALVAC vaccinations. Patient IGR38 had a prolonged hospitalization following appearance of grade 2 fever 9 hours after his second ALVAC vaccination. The temperature normalized after 24 hours. Patient CP71 had a prolonged hospitalization after dyspnea and fever appeared 2 hours after her first ALVAC vaccination. The symptoms disappeared after 24 hours. The patient received the next three ALVAC injections without subsequent fever. Patient MA38 had his first vaccination delayed because of a sepsis. The fever resolved after two days of antibiotherapy, which was maintained for two additional days. He was vaccinated the next day. Later that day, he experienced tachycardia and anxiety. The next days he developed dyspnea and fever, and died as a result of cardiac failure 4 days after the start of the treatment. No autopsy was performed and the precise cause of death was not determined. The most probable cause was sepsis. Patient LE1 was hospitalized for abdominal pain occurring 10 days after the third peptide vaccination. The pain was attributed to necrosis of abdominal metastatic lesions.

Tumor Responses

Forty patients were included in the study. All received at least one vaccination with ALVAC. Nine of them were removed because of disease progression before receiving four ALVAC vaccinations. Among the remaining 31 patients, including 30 with metastatic melanoma, 28 had disease progression. One melanoma patient, EB81, had a partial response, and two other melanoma patients, NAP34 and NAP35, had stable disease for more than 6 months. The clinical evolution of the 30 melanoma patients is summarized in Figure 4.

Even though they have no evident clinical benefit, mixed responses (ie, the regression of a subset of the metastases in patients with stable or progressive disease) deserve to be reported and described in detail because they may be important to understanding the relevance of the observed immune responses. A mixed response was observed in patients NAP34, CP67, LAU147, LAU624, and NAP33. Figure 5 displays the clinical evolution of the six melanoma patients who showed evidence of tumor regression. One notices that these regressions started several weeks after the onset of treatment and were often slow to proceed. Often, regressing lesions did not shrink simultaneously. In the

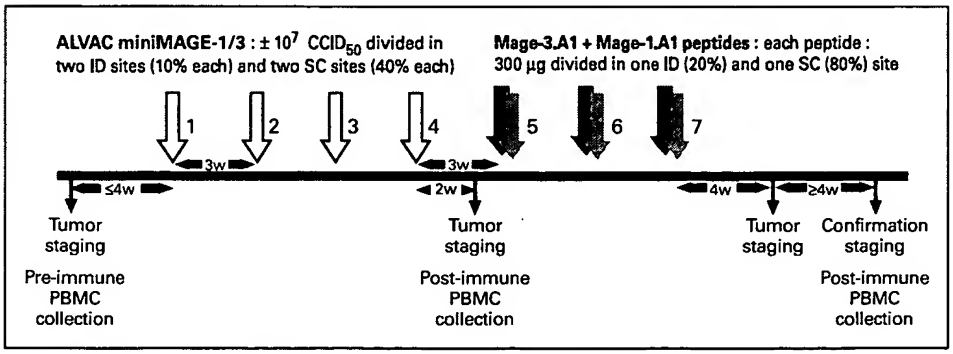

Fig 3. Vaccination schedule. $CCID_{50}$, 50% of the cell culture infectious dose; ID, intradermal; SC subcutaneous; w, weeks; PBMC, peripheral blood mononuclear cell.

Table 1. Patient Characteristics

Patients	Sex	Age	Tumor Type	Class I HLA A	Class I HLA B	Previous Treatments	Study Treatment: AJCC Stage* at Entry	Study Treatment: Total No. of Injections ALVAC	Study Treatment: Total No. of Injections Peptides†	Outcome: Tumor Response‡	Outcome: Survival (months)
Patients who received ≥ 4 ALVAC vaccinations (n = 31)											
EB81	F	72	MELA	A1, A2	B50, B57	Sg	III N2b	4	4 + 15	PR	> 58
NAP34	F	62	MELA	A11, A32	B35, B61	Sg	III N2b	4	4 + 6	SD (MxR)	> 41
NAP35	F	63	MELA	A1, A2	B35, B37	Sg	III N2b	4	4 + 4	SD	> 31
CP67	F	43	MELA	A1, A32	B8, B62	Sg, Ct, NSI	III N2	4	4 + 5	PD (MxR)	> 39
LAU147	F	44	MELA	A1, A24	B8, B49	Sg, NSI	IV M1a	4	0	PD (MxR)	> 26
LAU624	M	39	MELA	A1, A25	B17, B18	Sg	IV M1b	4	3	PD (MxR)	25
NAP33	F	65	MELA	A1, A31	B7, B57	Sg, Ct, NSI	III N2b	4	4 + 10	PD (MxR)	> 44
KUL73	F	71	MELA	A1, A2	B8	Sg, ILP	III N2b	4	0	PD	> 45
CP68	F	37	MELA	A1, A29	B44	Sg, NSI	III N2b	4	0	PD	4
LB2201	F	46	MELA	A11, A29	B35, B44	Sg, NSI	III N2c	4	3	PD	22
LB2196	M	73	MELA	A1, A3	B7, B35	Sg, NSI	III N2c	4	3	PD	24
NAP36	M	81	MELA	A1, A26	B8, B38	Sg	III N2c	4	1	PD	5
LY4	F	28	MELA	A11, A24	B18, B35	Sg, Ct, NSI	IV M1b	4	0	PD	12
CP69	F	76	MELA	A1, A2	B8, B27	Sg, Ct, NSI	IV M1a	4	2	PD	11
LB2268	F	86	MELA	A11, A28	B8, B35	Sg	IV M1a	4	0	PD	3
IGR37	M	45	MELA	A1, A3	B7, B8	Sg, Ct, NSI	IV M1b	4	1	PD	5
BB132	M	70	MELA	A1, A2	B8, B57	Sg	IV M1b	4	3	PD	7
LB2291	F	65	MELA	A1, A2	B7, B15	Sg, NSI, R	IV M1b	4	0	PD	13
VUB39	F	73	MELA	A1, A2	B7, B41	Sg, Ct	IV M1b	4	3	PD	10
LAU622	M	66	MELA	A1, A2	B7, B39	Sg	IV M1b	4	0	PD	12
LY5	F	48	MELA	A3, A23	B35, B50	Sg, Ct	IV M1b	4	1	PD	17
NAP37	F	28	MELA	A1, A2	B8, B62	Sg, Ct	IV M1b	4	0	PD	5
LAU407	M	56	MELA	A1, A2	B8, B44	Sg, Ct, NSI	IV M1b	4	3	PD	13
LG145	F	59	MELA	A24, A30	B13, B35	Sg, Ct, NSI	IV M1b	4	0	PD	5
HUG4	F	35	MELA	A1, A24	B8, B61	Sg	IV M1b	4	0	PD	2
UZG10	F	48	MELA	A1, A2	B15, B40	Sg, Ct	IV M1b	4	0	PD	11
LE1	F	63	MELA	A1, A26	B8, B55	Sg, Ct	IV M1b	4	3	PD	12
CP70	M	65	MELA	A2, A28	B7, B35	Sg, Ct, NSI	IV M1b	4	0	PD	3
LY6	M	57	MELA	A1, A24	B8, B61	Sg, Ct, NSI	IV M1b	4	0	PD	4
CP71	F	52	MELA	A23, A24	B35, B49	Sg, Ct, NSI	IV M1b	4	0	PD	3
MA39	M	43	NSCLC	A1, A32	B14, B47	Sg, Ct	IV M1	4	0	PD	5
Patients who received < 4 ALVAC vaccinations (n = 9)											
NAP38	F	45	MELA	A2, A25	B18, B35	Sg	III N2	2	0		12
NAP39	M	51	MELA	A1, A23	B44, B49	Sg	IV M1a	3	0		8
NAP40	M	33	MELA	A1, A24	B7, B18	Sg, NSI	III N1	3	0		8
IGR38	M	48	MELA	A1, A2	B37, B38	Sg, Ct	IV M1b	2	0		5
IGR39	M	69	MELA	A1	B8, B15	Sg, Ct	IV M1b	3	0		1
CF21	M	29	MELA	A1, A2	B62, B63	Sg, Ct, NSI	IV M1b	3	0		6
HUG5	M	57	MELA	A2, A3	B35, B51	Sg, NSI	IV M1b	3	0		9
MA38	M	51	H&N	A24, A32	B35	Sg, Ct	IV M1	1	0		<1
LB2282	M	47	NSCLC	A1, A19	B50, B51	Sg, Ct	IV M1	2	0		6

Abbreviations: AJCC, American Joint Committee on Cancer; MELA, melanoma; NSCLC, non–small-cell lung cancer; H&N, head and neck cancer; Sg, surgery; Ct, chemotherapy; NSI, nonspecific immunothrapy; ILP, isolated limb perfusion; R, radiotherapy. PR, partial response; SD, stable disease; PD, progressive disease; MxR, mixed response.

*AJCC, Cancer Staging Manual, 4th edition, 1992; Melanoma staging: III, regional disease, N (N2b, in-transit metastasis; N2c, metastasis > 3 cm in greater dimension in any regional lymph node[s] and in-transit metastasis); IV, distant metastasis, M (M1a, metastasis in skin or subcutaneous tissue or lymph node[s] beyond the regional lymph nodes; M1b, visceral metastasis). In-transit metastasis involves skin or subcutaneous tissue > 2 cm from the primary tumor not beyond the regional lymph nodes.

†Five patients who were thought by the investigator to have some benefit of the treatment received more than three vaccinations with the peptides, on a compassionate basis.

‡According to the WHO criteria. In addition, MxR were reported (ie, regression of a subset of the lesions in stable or progressive disease).

case of skin metastases, no local inflammation was noticed. These features are similar to those described in previous trials with MAGE peptides or protein.[3,4] A brief description of these six patients follows.

Patient EB81, who achieved a partial response, had many cutaneous in-transit metastases on her right leg at the onset of treatment that were progressing in size and number. Approximately 18 lesions were nodular, including some that were ulcerated, whereas the others were completely flat. All were pigmented (Fig 6). By the third ALVAC vaccination, the ulcerated nodules had dried out and flattened. Some large nodules increased in size, but became scabby. No new lesions had appeared. By the second peptide vaccination, flattening of almost all

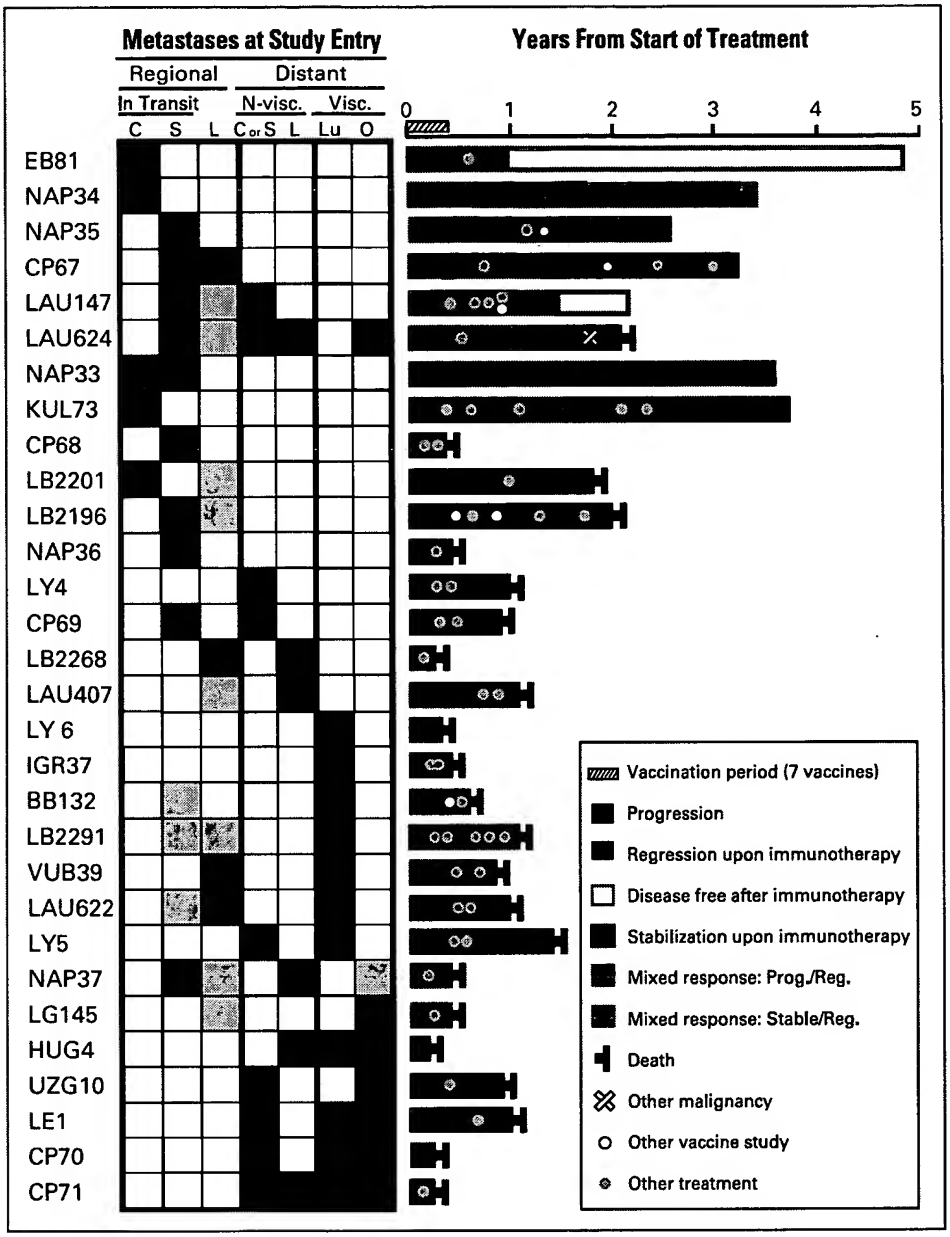

Fig 4. Outcome of the melanoma patients who received at least four vaccinations with ALVAC miniMAGE-1/3. Metastasis at study entry: Dark gray, measurable metastases at study entry; light gray, metastasis removed before study. n-visc., non-visceral distant metastasis; visc., visceral metastasis; C, cutaneous; S, subcutaneous; L, lymph node; Lu, lung; O, other visceral localization; Prog., progression; Reg., regression.

the nodular lesions was noted. A right inguinal adenopathy appeared, but no melanoma cells were observed in a fine-needle aspirate. The size of the adenopathy remained unchanged during 3 months, then increased and it was removed surgically. This lymph node was invaded by a large metastatic nodule, surrounded by a fibrotic shell infiltrated by lymphocytes and melanophages.[30] A right inguinal lymph node clearance was performed thereafter. None of the 10 removed lymph nodes was invaded by the tumor. The patient received additional vaccinations with the two peptides at increasing intervals. Some remaining pigmented patches were excised and were found to contain melanophages, but no tumor cells. The melanin tattooing disappeared very slowly. Patient EB81 had no evidence of disease 1 year after treatment onset and has remained disease-free for more than 4 years. During that period, she has received several vaccinations with either the MAGE-3.A1 peptide or the ALVAC virus for the purpose of analyzing their impact on the frequency of anti-vaccine CTL.[23]

NAP34, one of the two patients who achieved disease stabilization, showed regression of one in-transit metastasis of the leg and stabilization of 11 others following vaccination. This was followed by slow disease progression, then by further regression of most in-transit metastases.

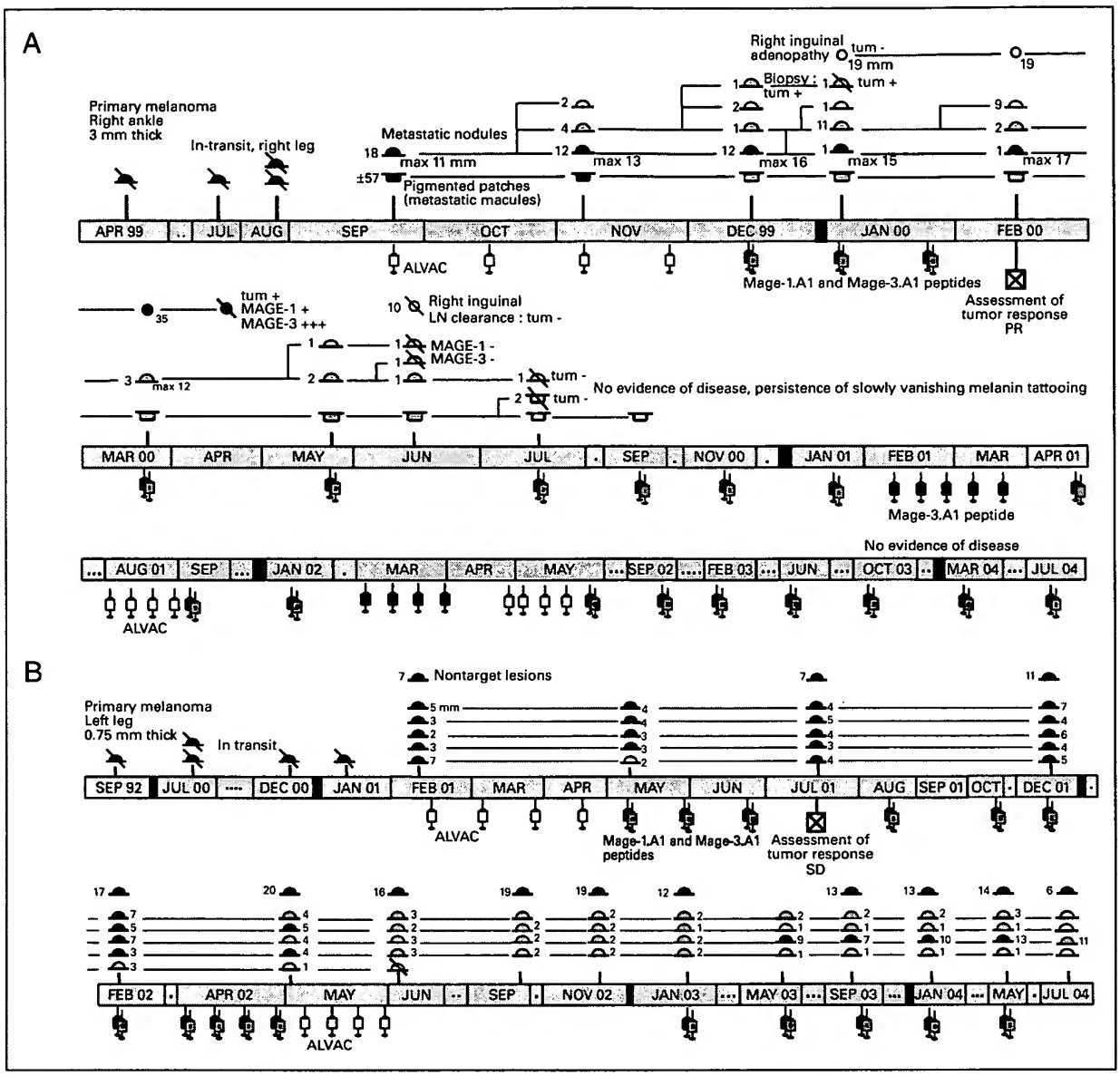

Fig 5. Course of the disease of the patients who experienced tumor regression. (A) Patient (PT) EB81; (B) PT NAP34; (C) PT CP67; (D) PT LAU147; (E) PT LAU624; (F) PT NAP33. IFN-α, interferon alfa; DTIC, dacarbazine; CDDP, cisplatin; tum +, tumor cells visible histologically; tum –, tumor cells not visible; MAGE-3 +, expression of gene MAGE-3; MAGE-3 –, no expression of gene MAGE-3; PR, partial response; SD, stable disease; PD, progressive disease; MxR, mixed response.

A mixed response was seen in four patients with disease progression. In patient CP67, vaccination was associated with regression of regional subcutaneous and lymph node metastases. Eventually, all four skin nodules disappeared completely, but the adenopathy increased in size and new metastases appeared. Patient LAU147 had only one regional subcutaneous metastasis, documented by cytologic examination, present at study entry. This nodule regressed completely during vaccination, but two new metastases appeared, one in the breast and one in the brain, and were removed surgically. New metastases appeared thereafter, and were treated initially by surgery, then by a combination of chemotherapy with temozolomide and vaccination with the MAGE-3.A1 and MAGE-3.A24 peptides associated with the immunologic adjuvant Montanide ISA 51(Seppic, Paris, France). A complete response was obtained with the latter treatments, and has been maintained for more than a year. Patient LAU624 experienced regression of four distant subcutaneous nodules following vaccination, but two distant lymph node and one spleen metastases showed progression. Patient NAP33 had a slow regression of three SC in-transit nodules. Two of them disappeared completely, while the third remained detectable only by ultrasound for many months. Then it increased in size again, and new in-transit nodules appeared.

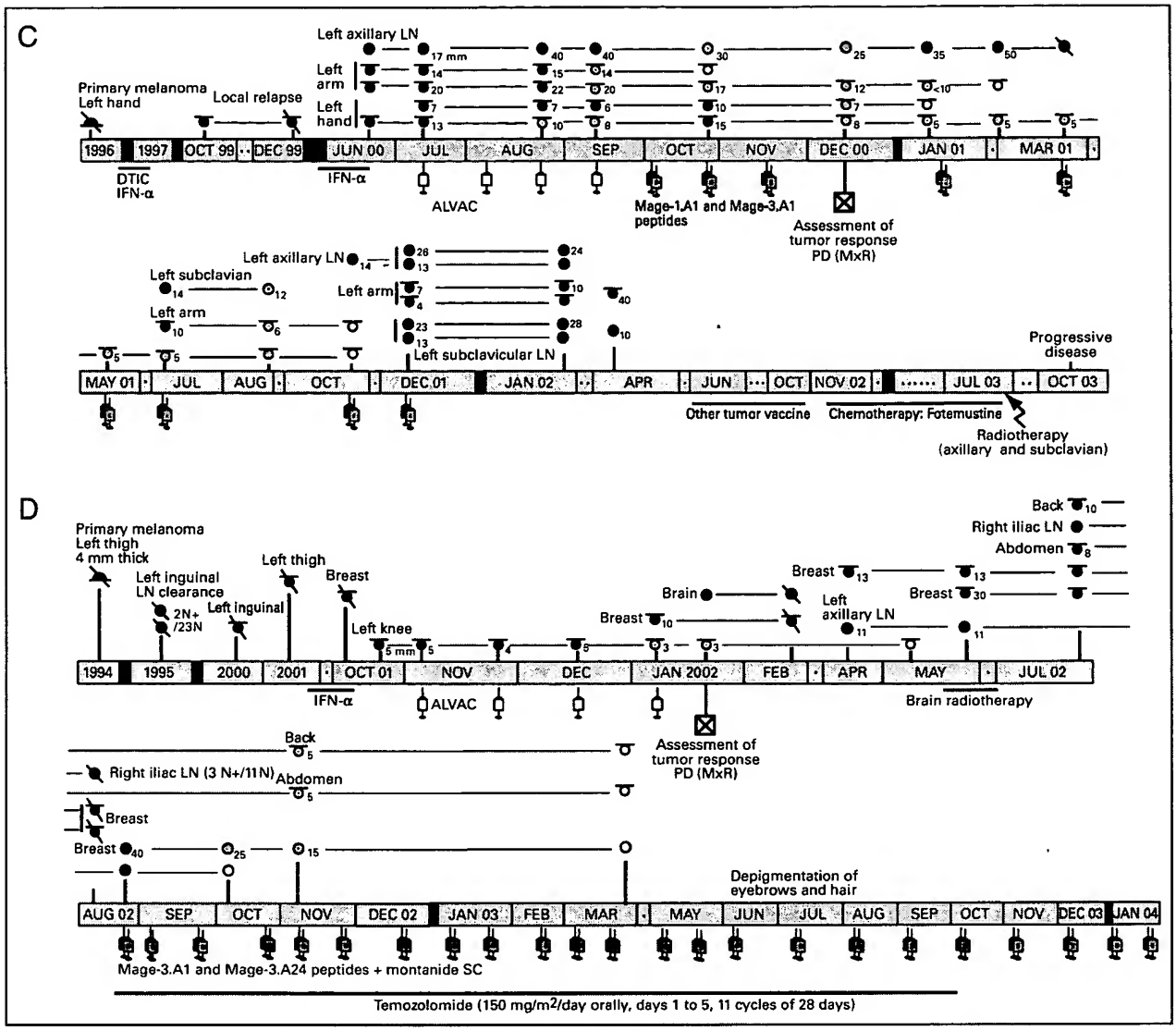

Fig 5. (continued)

Immunologic Responses to the Vaccine Antigens

Frozen PBMCs and serum collected before and after ALVAC vaccination were available for immunologic tests from 29 of the 40 enrolled patients. Nine of them also had immunologic material collected after the three peptide vaccinations.

The CTL response against the MAGE-3.A1 and MAGE-1.A1 vaccine antigens was evaluated with a sensitive approach which measures CTLp frequencies as low as 8×10^{-7}. This approach involves the stimulation of blood lymphocytes repeatedly with the antigen during 3 weeks in microwell plates under limiting dilution conditions, followed by the staining of responder cells with an A1/MAGE-3 or A1/MAGE-1 tetramer, and detection of the positive microcultures by flow cytometry. The CTLp frequency is deduced from the proportion of positive wells. Importantly, individual tetramer-stained CTL are cloned and the lytic activity of the CTL clones is verified to be specific for the MAGE antigens. The T cell receptor genes are sequenced so as to distinguish different clonotypes recognizing the same antigen.

A detailed account of this MLPC/tetramer/cloning approach and a description of the MAGE-3.A1 CTL responses in 17 patients from the present trial, selected according to the availability and quality of frozen PBMCs, and including four patients with evidence of tumor regression, have been reported previously.[7,23] A synthesis of these results extended to the MAGE-1.A1 antigen is shown in Table 2. Two patients, VUB39 and NAP37, were found to have a preexisting CTL response against the MAGE-3.A1 antigen. Since this precluded the demonstration of a response triggered by the vaccine candidate, they were excluded from the analysis. In the remaining 15 patients, we observed a

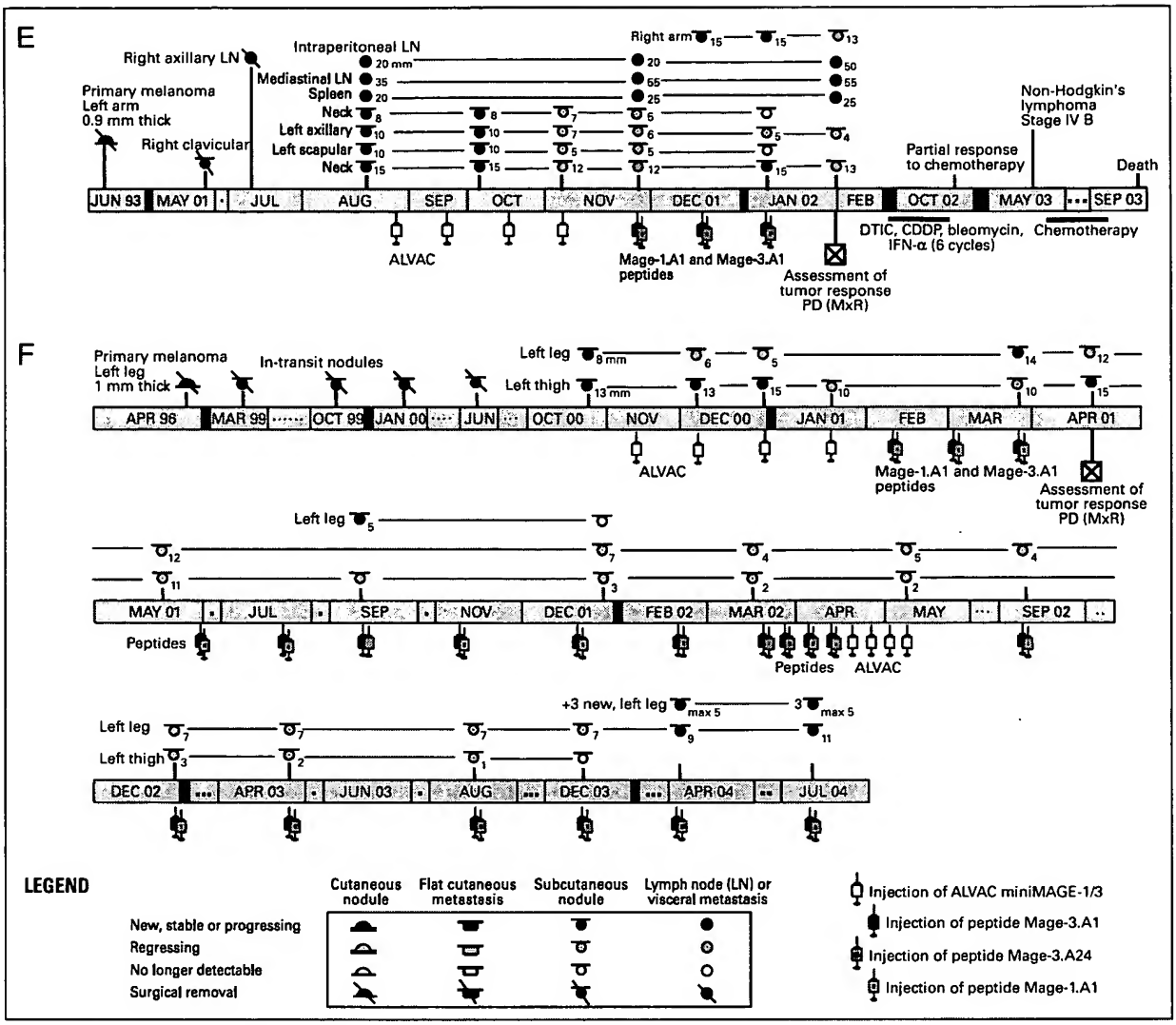

Fig 5. (continued)

significant correlation between anti-MAGE-3.A1 CTL response and the occurrence of tumor regressions: 3 of 4 patients with tumor regression and only 1 of 11 patients without regression mounted a detectable CTL response (Fisher's exact test, $P = .033$). These responses were detectable in the blood collected after ALVAC vaccination and before peptide vaccination in all positive patients. No anti-MAGE-1.A1 CTLp could be detected in the postimmune blood of 12 HLA-A1 patients, including four with tumor regression.

Antigenic peptides MAGE-$3_{168\text{-}176}$ and MAGE-$1_{161\text{-}169}$ are also presented by HLA-B*3501. Three patients carrying the HLA-B*3501 allele, NAP34, NAP35 and LB2196, were evaluated for their CTL response. No CTL response was detected after four ALVAC vaccinations (data not shown). However, further vaccination of patient NAP34 with the two peptides and additional booster vaccinations with ALVAC were associated with the appearance of an anti-MAGE-1.B35 CTL clone in her blood (data not shown). This weak CTL response might be relevant, because this patient experienced tumor regressions that started late after the onset of the vaccinations and proceeded quite slowly. Patients with B35 alleles other than B*3501 were not analyzed, because the appropriate HLA/peptide tetramers were not available.

CTL responses against vaccine-encoded tumor antigens were also assessed in 27 evaluable patients by IFN-γ ELISPOT. No significant increase in IFN-γ–secreting T cells was detected after exposure to the MAGE-3 and MAGE-1 peptides (data not shown). These results are consistent with the results of the MLPC/tetramer/cloning assay, as all the CTLp frequencies measured by this latter assay were below the 10^{-4} detection limit of ELISPOT, except for LAU147. PBMCs from this patient, however, were not available for ELISPOT analysis.

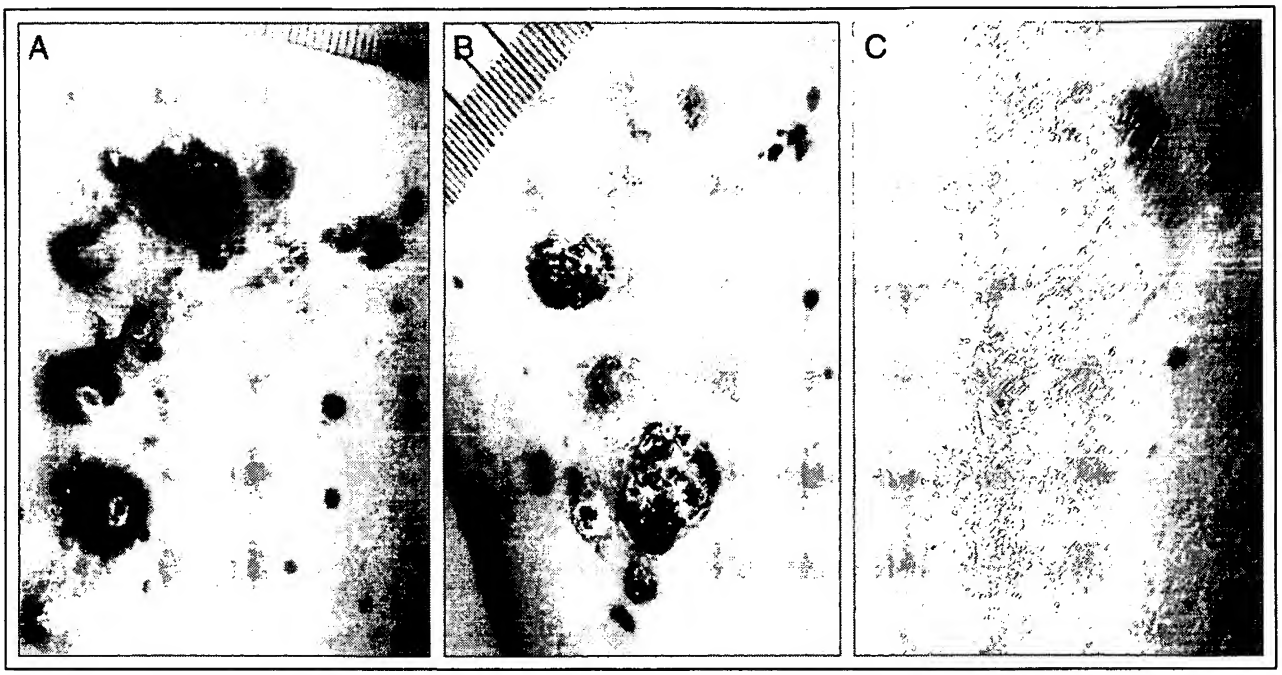

Fig 6. Evolution of skin metastases on the leg of patient EB81 during treatment. (A) Before vaccination; (B) after four vaccinations with ALVAC (after 3 months); (C) after 10 months.

ELISA was used to monitor vaccine induced antibody responses against the wild-type ALVAC virus. Consistent with previous reports involving vaccination with ALVAC, an antibody response against the virus was detected in the serum of all patients (data not shown). No antibody response was found against the MAGE-$3_{57\text{-}219}$ and MAGE-$1_{57\text{-}219}$ polypeptides, which contain the epitopes expressed by the ALVAC-based vaccine candidate (data not shown).

DISCUSSION

This clinical trial establishes a good safety profile for vaccination with a recombinant ALVAC virus expressing a MAGE-1/3 minigene. The reported adverse reactions were in line with previous clinical reports on the toxicity of various ALVAC recombinants.[10-14] Moreover, ALVAC was well tolerated after ID injection, which had not been investigated before in humans.

Our study also shows that this vaccine has a weak antitumor effectiveness. Among the 30 melanoma patients who received four ALVAC vaccinations, only one partial response and two stabilized diseases were recorded. The clinical efficacy of the experimental vaccine may be underestimated due to the rather short observation period, because two patients, NAP34 and NAP33, experienced regression of most tumor lesions after their tumor response had been evaluated as stable disease and progressive disease, respectively. In both cases, these regressions persisted for more than two years, in the absence of any other treatment. Our clinical data do not allow to conclude that the ALVAC vaccine candidate is either superior or inferior to other vaccine modalities in terms of antitumor effectiveness, as similarly low rates of tumor response were observed with the MAGE-3.A1 peptide and the MAGE-3 protein (W.H.J. Kruit, unpublished observations).[3,4] Other vaccine modalities with different tumor antigens have met with similarly low clinical success.[31,32]

We have considered three principal potential causes for the failure of a cancer vaccine to induce tumor regression. The vaccine might fail to induce a CTL response. The activated CTL might fail to reach the tumor sites. Finally, tumor resistance or local immunosuppression might prevent the anti-vaccine T cells from attacking the tumor cells. As a first step in evaluating these causes for failure, we have engaged in a detailed analysis of the T cell responses in vaccinated patients.

We first investigated whether our vaccine candidate had induced anti-vaccine CTL responses, ie, CTL directed at the MAGE-3 and MAGE-1 antigens. Preliminary work had shown that these CTL responses would usually be too low to be evaluated by the usual CTL monitoring techniques such as the commonly performed ELISPOT tests or the direct tetramer and intracellular cytokine assays. Accordingly, the pre- and postimmunization CTL precursor frequencies were measured with a sensitive monitoring approach, involving limiting dilution in vitro restimulation, tetramer analysis, and cloning.[23] Using this approach, a CTL response was detected in four of 17 evaluated HLA-A1 patients. With one exception, these CTL responses provided a frequency of CTL precursors in the blood that

Table 2. CTL Responses Against MAGE-3.A1 and MAGE-1.A1 In 17 HLA-A1 Patients Vaccinated With ALVAC Mini-MAGE-1/3 Including Four Patients Who Experienced Tumor Regression

Patients	CTL Responses	
	MAGE-3.A1	MAGE-1.A1
Patients with tumor regression (n= 4)		
EB81	+	–
CP67	–	–
LAU147	+	–
NAP33	+	–
Total	3 of 4	0 of 4
Patients without tumor regression (n=13)		
NAP35	–	–
KUL73	–	–
LB2196	–	–
NAP36	+	–
CP69	–	nt
IGR37	–	nt
BB132	–	–
VUB39	P	–
LAU407	–	–
NAP37	P	nt
LAU622	–	nt
UZG10	–	–
Total	1 of 11	0 of 8

NOTE. A CTL response (+) is defined as a postimmune CTLp frequency above 4×10^{-6} with a preimmune value below 8×10^{-7}, and/or as the occurrence of at least one repeated CTL clone in the postimmune, but not in the preimmune blood, even with a CTLp frequency below 4×10^{-6}. A clone is said to be repeated if it is found at least three times in a set of fewer than 10 independent CTL clones, or four times in a set of fewer than 20 independent CTL clones.[7] (–) indicates no CTL response.
Abbreviations: CTL, cytolytic T lymphocyte; CTLp, CTL precursor; nt, not tested; P, pre-existing CTL response, already present before vaccination (patient not assessable).

was lower than 10^{-5} of CD8 T cells. We conclude that the ALVAC vaccine has a weak capacity to trigger anti-MAGE CTL responses. It is noteworthy that all the observed CTL responses were directed against the MAGE-3.A1 antigen. Our in vitro analysis of the immunogenicity of the viral vaccine candidate showed that both the MAGE-3.A1 and the MAGE-1.A1 antigens were appropriately processed by infected dendritic cells. However, the presenting cells were better recognized by the anti-MAGE-3.A1 than by the anti-MAGE-1.A1 CTL clone, raising the possibility that MAGE-3.A1 peptide was better processed. This may explain the preferential CTL response observed in this trial.

Notwithstanding their weakness, the observed CTL responses appear to be significant, because they are correlated with clinical evidence of tumor regression. Three of four evaluated patients who showed evidence of tumor regression had a CTL response, as opposed to only one of 11 patients without regression. For this type of analysis, aimed at generating hypotheses regarding the process of tumoral regression following vaccination as opposed to assessing therapeutic efficacy, we strongly feel that it is important to take into account all instances of observed tumor regression, whether or not they qualify as objective responses. It is noteworthy that there was no correlation between the magnitude of the observed vaccine-induced CTL response and that of the clinical response. NAP33 had an almost undetectable CTL response, but has remained free of active disease for more than 2 years, whereas LAU147 demonstrated major disease progression despite a strong CTL response.

For the four detectable CTL responses, analysis of T-cell receptor usage indicated that these responses were monoclonal.[7,23] The CTL response that had been observed in a patient vaccinated with the MAGE-3.A1 peptide without adjuvant was also monoclonal.[6] On the other hand, in a small series of patients vaccinated with autologous dendritic cells pulsed with the MAGE-3.A1 peptide, polyclonal CTL responses were observed.[33]

To try to understand the paradox of observing tumor regression in patients with a low level of antivaccine T cells in the blood, we examined in patient EB81 the frequency of these T cells inside various metastases. We felt that a high enrichment of these T cells relative to other T cells might solve the paradox. But little enrichment was observed.[30] Accordingly, we considered it unlikely that antivaccine T cells could be the sole specific effectors of the complete rejection of the skin metastases of this patient. We examined whether T cells could be found against other antigens borne by the tumor, and we found them at the remarkably high frequency around 10^{-3} of blood CD8 T cells (ie, approximately 1,000 times higher than that of the vaccine T cells).[34] The same finding was made in four other melanoma patients. Remarkably, these T cells, which were labeled antitumoral as opposed to the antivaccine T cells, were already present at high frequency in all these patients before vaccination. In patient EB81, most of the antitumoral T cells were directed against various antigens encoded by another gene of the MAGE family, namely MAGE-C2. In patient EB81, the anti-tumor T cells showed enormous enrichment in the tumor, with some anti-MAGE-C2 CTL clones amounting to about 5% of the CD8 T cells present in the tumor.[30] Similar observations have been made recently on a patient vaccinated with dendritic cells (A. Van Pel, unpublished observations).

The presence of tumor-infiltrating lymphocytes (TILs) was reported many years ago by several groups.[35-37] Moreover, some of these TILs were shown to be effective, after in vitro amplification, for adoptive transfer T-cell therapy in melanoma patients.[38,39] TILs may slow tumor evolution in a number of patients, constituting a partially effective form of "immunosurveillance." Possibly, they may even eliminate some early tumors altogether. But this spontaneous response clearly becomes ineffective at one stage in the patients whose disease progresses.

On the basis of our findings, we favor the following scenario for the elimination of the tumor that occasionally follows vaccination. Before vaccination, the tumor and the blood contain a high level of antitumor T cells. These cells have become ineffective, even though they can easily be reawakened by in vitro restimulation with tumor cells

in the presence of IL-2. In some patients, vaccination produces CTL that reach the tumor and can resist the local immunosuppression long enough to attack some tumor cells. This results in a focal reversal of the immunosuppressive environment. This in turn enables the reawakening of old antitumor CTL clones or the generation of new antitumor CTL clones. These active antitumor CTLs expand in much larger numbers than the antivaccine CTLs and they are responsible for the elimination of the bulk of the tumor cells. In other terms, the antivaccine CTL serve only as a spark that reignites the bulk of the antitumor T cell response. Our results suggest that this spark generates new antitumor T cells, as new dominant T-cell receptor clonotypes appear after vaccination. Whether some previously present dormant T cells are reactivated remains an open question.

The rejection scenario proposed in the preceding paragraphs has several implications. One is that tumor escape due to the selection of tumor variants that have lost the expression of the vaccine antigen may not be a limiting factor for the efficiency of antitumor vaccination, because such antigen-loss variants would still be sensitive to the many CTLs directed against other antigens of the tumor. Admittedly, loss of all HLA expression could have farther-reaching consequences, but these cells ought to be hypersensitive to natural killer cells.[40] Another major implication is that vaccination might be combined usefully with treatments that alleviate the local immunosuppression of the tumor. One of many possibilities is to deplete regulatory T cells before vaccination.[38] Selective usage of cytokines concomitant to vaccination might be effective also.

It is possible that some T-cell clones have functional properties that render them more capable of serving as a spark by resisting the immunosuppressive environment of the tumor. Considering that many of our responses are monoclonal, it may be useful to vaccinate with a larger number of different antigens in order to increase the chances of obtaining a T-cell clone with the optimal functional properties. Finally, the frequency of antivaccine T cells may also be a limiting factor. Accordingly, we will try to vaccinate patients with higher doses of the ALVAC-MAGE vaccine candidate, because our in vitro studies indicated that injection of dendritic cells with higher doses of virus generated a higher CTL activation.

Authors' Disclosures of Potential Conflicts of Interest

Although all authors completed the disclosure declaration, the following authors or their immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Impact of the Number of Treatment Courses on the Clinical Response of Patients Who Receive High-Dose Bolus Interleukin-2

By Kimberly R. Lindsey, Steven A. Rosenberg, and Richard M. Sherry

Purpose: **To determine the impact of treatment with successive courses of high-dose bolus interleukin-2 (IL-2) on the incidence of clinical responses in patients with metastatic melanoma or renal cell cancer.**

Patients and Methods: **A consecutive series of 350 patients with either metastatic melanoma or renal cell cancer who were treated with high-dose bolus IL-2 in the Surgery Branch, National Cancer Institute, between September 1985 and November 1996 was analyzed, with a median potential follow-up of 7.1 years. All patients were treated with 720,000 IU/kg of IL-2 administered by a 15-minute intravenous infusion every 8 hours for up to 5 days, as clinically tolerated per cycle. Patients were retreated according to clinical response and tolerance to the IL-2 therapy.**

Results: **Of the 149 patients with melanoma, 10 achieved complete responses (CRs) and 13 partial responses (PRs), for an overall response rate of 15.4%. Of the 201 patients with renal cell cancer, 18 achieved CRs and 20 PRs, for an overall response rate of 19.0%. Among responding patients, 21 of 23 with melanoma and 34 of 38 with renal cell cancer developed at least PRs after the first course of IL-2.**

Conclusion: **Most patients with metastatic melanoma and renal cell cancer who achieved PRs or CRs to intravenous high-dose bolus IL-2 were identified after the first course of therapy. Those who demonstrated no response after two treatment courses failed to respond to additional IL-2 therapy. Based on this retrospective analysis, we recommend that patients who exhibit objective responses to treatment with high-dose bolus IL-2 receive additional treatment courses until either CR or IL-2 intolerance develops. Patients who do not achieve objective responses after two courses of IL-2 should receive no further treatment with this regimen.**

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PATIENTS WITH metastatic melanoma or renal cell cancer have a median survival of less than 1 year, and in the absence of effective treatment, almost all eventually succumb to their disease. The administration of interleukin-2 (IL-2) causes complete responses (CRs) or partial responses (PRs) in approximately 15% of patients with metastatic melanoma and in 19% of patients with renal cell cancer.[1] Approximately one half of these represent durable CRs. In May 1992, IL-2 was licensed by the Food and Drug Administration for use in the treatment of patients with metastatic renal cell cancer. IL-2 was approved for treating patients with metastatic melanoma in February 1998.

At the National Cancer Institute, Surgery Branch, we began to treat patients with high-dose bolus recombinant IL-2 alone in September 1985.[2-4] Since that time, 350 consecutive patients have been treated with this regimen. These patients were observed for a median of 7.1 years as of March 1998. The longest CR, which was ongoing at the time of this writing, was 12.4 years.[1] This unique population was analyzed to determine the tempo of the clinical responses in patients treated with high-dose bolus IL-2 alone to identify the antitumor effects of successive courses of immunotherapy. In addition, because IL-2 can be associated with significant toxicity, we hoped to elucidate a reasonable strategy for patient treatment in this clinical setting.

PATIENTS AND METHODS

Patients

The study population consisted of a consecutive series of 350 patients treated at the Surgery Branch, National Cancer Institute between September 1985 and November 1996. All patients had clinically progressive metastatic renal cancer or melanoma and had received no other therapy for at least 30 days before entering onto the treatment protocol. The protocols were approved by the Institutional Review Board of the National Cancer Institute, and all patients provided informed consent. Response to treatment and survival were continuing to be assessed in all patients as of March 1998, with a median potential follow-up of 7.1 years.

Patients who had received prior IL-2 or who had evidence of concomitant severe respiratory, cardiovascular, or renal disease were not accepted into these trials. Before entry onto the protocol, all patients were evaluated with computed tomographic (CT) or magnetic resonance imaging scans of the brain, CT scans or full-lung tomograms of the lungs, abdominal CT scans, and radionuclide bone scans. Patients were not eligible if they had CNS metastases. All participants

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Table 1. Patient Characteristics

	Melanoma		Renal Cell		Total	
	No. of Patients	%	No. of Patients	%	No. of Patients	%
Total patients	149	100	201	100	350	100
Sex						
Male	99	66	138	69	237	68
Female	50	34	63	31	113	32
Age group						
11-20 years	3	2	1	0	4	1
21-30 years	21	14	10	5	31	9
31-40 years	52	35	27	13	79	23
41-50 years	37	25	69	34	106	30
51-60 years	25	17	71	35	96	27
61-70 years	11	7	23	11	34	10
Race						
Asian	0	0	1	0	1	0
Black	1	1	8	4	9	3
Hispanic	0	0	2	1	2	1
Other	4	3	8	4	12	3
White	144	97	182	91	326	93
Performance status						
0	117	79	146	73	263	75
1	26	17	44	22	70	20
2	6	4	10	5	16	5
3	0	0	1	0	1	0
Prior therapy						
None	2	1	7	3	9	3
Surgery	144	97	192	96	336	96
Chemotherapy	39	26	13	6	52	15
Radiotherapy	20	13	18	9	38	11
Hormonal	1	1	7	3	8	2
Immunotherapy	60	40	32	16	92	26
Any two or more	79	53	54	27	133	38
Any three or more	31	21	14	7	45	13

in the trial underwent stress ECG or stress radionuclide ejection or thallium scans, except for a few patients who were entered early onto the protocol. Patients with evidence of ischemic heart disease or significant arrhythmias were not eligible.

Treatment

Recombinant IL-2 (supplied by Cetus Oncology Division, Chiron Corporation, Emeryville, CA) was administered intravenously over 15 minutes at a dose of 720,000 IU/kg. IL-2 was reconstituted from a lyophilized powder with 1.2 mL of sterile water per vial. Vials also contained 5% mannitol and approximately 130 mg of sodium dodecyl sulfate per milligram of IL-2. A dilution of IL-2 in 50 mL of normal saline containing 5% human serum albumin was used for infusion. Patients received IL-2 every 8 hours. Patients with evidence of stable or responding disease were eligible to receive a second course of treatment. IL-2 was routinely administered in a general surgery ward, although some patients required transfer to the intensive care unit for monitoring or administration of vasopressors. All patients received medications such as acetaminophen and indomethacin to prevent the side effects associated with IL-2 administration.[4,5]

Evaluation of Response

Metastatic tumor deposits were measured with either radiologic studies or physical examination, and the product of maximal perpendicular tumor diameters was calculated. Measurements were taken before treatment, 2 months after treatment, and at regular intervals thereafter. A PR was defined as a 50% or greater reduction in the sum of products of the perpendicular diameters of all lesions that lasted at least 1 month with no new or growing lesions. A CR was defined as the complete disappearance of all disease without the appearance of any new disease for at least 1 month. A minor response (MR) was defined as having a 25% to 49% reduction in tumor burden. Anyone who did not achieve at least a 25% reduction in disease was considered to have no response to treatment. Response and survival durations were calculated from the time of the first dose of IL-2.

RESULTS

Patient Characteristics

Between September 1985 and November 1996, 350 patients (149 with metastatic melanoma and 201 with metastatic renal cell cancer) received therapy with high-dose bolus IL-2 in the Surgery Branch, National Cancer Institute (Table 1). Most patients ranged between the ages of 21 and 60 years. The male-to-female ratio was approximately 2:1. The study population was heavily pretreated.

Table 2. Response of Patients Treated with High-Dose Bolus IL-2

Diagnosis	Total No. of Patients	CR		PR		Total Responses	
		No. of Patients	%	No. of Patients	%	No. of Patients	%
Melanoma	149	10	6.7	13	8.7	23	15.4
Renal cancer	201	18	9.0	20	10.0	38	19.0
Total	350	28		33		61	17.4

Thirty-eight percent of the patients had received two or more treatments each for their cancer, and 13% had received three or more different treatments each.

Response to Therapy

The response rates to high-dose IL-2 in the study population are presented in Table 2. Of the 149 melanoma patients, 10 achieved CRs and 13 PRs, for an overall response rate of 15.4%. Of the 201 patients with renal cell cancer, 18 experienced CRs and 20 PRs, for an overall response rate of 19.0%. Thus, of the 350 patients, 28 achieved CRs and 33 PRs, for an overall response rate of 17.4%.

Tempo of Response

Patient responses after the administration of each course of IL-2 for patients with melanoma or renal cell cancer are presented in Tables 3 and 4. For courses 2 through 4, patients are listed according to their clinical response to prior courses of therapy. In the melanoma cohort (Table 3), 21 of 149 patients developed clinical responses (18 PRs and 3 CRs) to the first course of therapy. Seventeen patients who achieved PRs to course 1 went on to receive a second course of therapy. Among these, one patient developed a CR. Forty-three patients who showed no response to course 1 of IL-2 received a second course of therapy, and two of these developed PRs. Eight patients who showed no response to courses 1 and 2 of IL-2 received a third course of therapy, and none of these patients responded. Eleven patients who achieved PRs after two courses of IL-2 received a third course of therapy, and one of these patients developed a CR. All 23 patients who sustained objective responses to high-dose IL-2 developed at least PRs after two courses of therapy.

In the renal cell cancer cohort (Table 4), 34 of 201 patients achieved objective responses (30 PRs and 4 CRs) and one developed an MR to the first course of therapy. Twenty-eight patients who achieved PRs to course 1 received a second course of therapy, with three of these developing CRs. The patient who sustained an MR achieved a PR after course 2 of high-dose IL-2. Sixty-three patients who showed no response to course 1 received a second course of therapy, and two of these developed PRs and one an MR. Nine patients who had not responded to two courses received a third course, and none developed an objective response. The one patient with an MR after two courses was retreated and achieved a CR. Twenty-one patients who had PRs after two courses of IL-2 received a third course of therapy, and eight of these developed CRs. Seven patients with PRs after three courses of therapy received a fourth course of treatment, and two of these achieved CRs. All but one of the 38 patients with renal cell cancer who sustained objective responses to high-dose IL-2 had developed at least PRs after two courses of therapy.

The duration of CR was analyzed in both melanoma and renal cell cancer. The duration of CR was not related to whether a CR was attained after the first course of IL-2 or

Table 3. Response to Successive Courses of High-Dose IL-2 in Patients With Metastatic Melanoma

	Response to Course 1			
No. of patients treated with course 1 (n = 149)	NR 128	MR 0	PR 18	CR 3
	Response to Course 1			
No. of patients treated with second course (n = 63)	NR 43		PR 17	CR 3
Response to courses 1 and 2				
NR	41		—	—
MR	—		—	—
PR	2		16	—
CR	—		1	3
	Response to Courses 1 and 2			
No. of patients treated with third course (n = 20)	NR 8		PR 11	CR 1
Response to courses 1-3				
NR	8		—	—
MR	—		—	—
PR	—		10	—
CR	—		1	1
	Response to Courses 1-3			
No. of patients treated with fourth course (n = 4)	NR 1			PR 3
Response to courses 1-4				
NR	1			—
PR	—			3
CR	—			—

Abbreviation: NR, no response.

Table 4. Response to Successive Courses of High-Dose IL-2 in Patients With Metastatic Renal Cell Cancer

	Response to Course 1			
No. of patients treated with course 1 (n = 201)	NR 166	MR 1	PR 30	CR 4
	Response to Course 1			
No. of patients treated with second course (n =96)	NR 63	MR 1	PR 28	CR 4
Response to courses 1 and 2				
NR	60	—	—	—
MR	1	—	—	—
PR	2	1	25	—
CR	—	—	3	4
	Response to Courses 1 and 2			
No. of patients treated with third course (n = 38)	NR 9	MR 1	PR 21	CR 7
Response to courses 1-3				
NR	9	—	—	—
MR	—	—	—	—
PR	—	—	13	—
CR	—	1	8	7
	Response to Courses 1-3			
No. of patients treated with fourth course (n = 10)	NR 1		PR 7	CR 2
Response to courses 1-4				
NR	1		—	—
PR	—		5	—
CR	—		2	2

after subsequent courses of IL-2 therapy. All patients who experienced PRs ultimately progressed. The median durations of PR for patients with metastatic melanoma and those with renal cell cancer were 21 and 36 months, respectively. In contrast, patients who achieved CRs had continued, ongoing responses at 21 to 162 months for melanoma and at 36 to 147 months for renal cell cancer. Factors that might predict or be associated with CR among patients treated with high-dose IL-2 have been analyzed previously.[1] Prior immunotherapy adversely affected the chances of achieving a CR. Five of the 28 patients who achieved CRs in this study had received two or more therapies before high-dose IL-2 alone. The median duration of response for this group was 84 months (range, 46 to 147 months). Two or more previous therapies had been given to eight of 33 patients who achieved PRs to high-dose IL-2 alone. The median duration of response for this group was 36 months (range, 8 to 142 months).

Indications for Discontinuing Treatment

In these 350 patients, there were three treatment-related deaths. Table 5 lists the reasons for discontinuing treatment after each of the four courses. The majority of patients

Table 5. Reasons for Discontinuing Treatment With IL-2 in Patients With Melanoma and Renal Cell Cancer

	No. of Courses Completed Before Discontinuation (no. of patients)			
Reason	1	2	3	4
Progressive disease	176	77	17	6
IL-2 toxicity	8	5	1	1
Death	3			
CR		4	19	1
Other	4	15	6	7

developed progressive disease at some point during treatment. Once this occurred, treatment was stopped. Several patients developed severe IL-2 toxicity that was not easily reversed by supportive measures, and these patients were not retreated. These toxicities included mental status changes that required intubation, severe cardiac arrhythmias, and severe renal dysfunction. The cardiopulmonary,[3,6-13] renal,[3,6-9,14-17] and hematologic[3,6-9,18,19] toxicities associated with IL-2 administration have been described in detail.

The reasons for discontinuing treatment listed as "Other" in Table 5 included IL-2 intolerance to the reversible non–life-threatening side effects (eg, nausea and diarrhea) and patient refusal. Classified in this category were 32 patients for whom the reason for discontinuation of therapy could not be ascertained from the medical records.

DISCUSSION

The objective response rate of patients with metastatic melanoma or renal cell cancer treated with high-dose bolus IL-2 has been reported in most series to be 15% to 25%, with 5% to 10% of patients achieving CRs.[1] Clinical experience with this regimen over the last decade has led to a reduction in the morbidity associated with this therapy.[3,6] Although our expertise in the administration of IL-2 has improved, there is little information regarding the broader clinical issues concerning when to discontinue therapy for patients with stable metastatic disease. Furthermore, there is no established guideline for how long to continue consecutive courses of therapy for patients who show responses to treatment. This retrospective analysis focused on the tempo of clinical responses to address these issues.

The response rate to IL-2 in the melanoma cohort was 15.4% (10 CRs and 13 PRs). Among these 23 patients, 21 achieved at least PRs after the first course of therapy. The response rate in the renal cell cancer cohort was 19.0% (18 CRs and 20 PRs). Among these 38 patients, 34 achieved at least PRs and one developed an MR after the first course of therapy. Thus, in both histologic cohorts, the preponderance

(90%) of patients who responded to this regimen were identified after one course of therapy.

It is known that, for patients with renal cell cancer or melanoma who respond to high-dose IL-2, CRs are usually durable, whereas patients with PRs eventually progress.[1] Our strategy for patients who developed PRs to IL-2 was to offer additional courses of IL-2 as tolerated, provided that there was evidence of stable or regressing disease. We also gave a consolidation course after a CR was achieved. The outcome of successive courses of therapy for patients with melanoma who developed PRs and then received additional courses of therapy is noted in Table 3. One of the 17 patients with melanoma who had developed PRs to course 1 achieved a CR to course 2 of IL-2, and one of 11 patients with PRs after the second course of therapy achieved a CR after course 3 of IL-2. The outcome for patients with renal cell cancer who developed PRs and then received additional courses of therapy is noted in Table 4. Three of the 28 patients with renal cell cancer who developed PRs to course 1 achieved CRs in course 2 of IL-2, and eight of 21 patients who had PRs after the first and second courses of therapy achieved CRs after course 3 of IL-2. Two of the seven patients with PRs after three courses of therapy achieved CRs after the fourth course of IL-2. Because of the strategy used to determine which patients were retreated, we could not determine whether those patients with PRs would have achieved CRs even if no further treatment had been given.

The outcome for patients who showed no response to IL-2 and subsequently received additional IL-2 was analyzed according to the course of therapy. (Tables 3 and 4). Forty-three patients in the melanoma cohort who had no response to course 1 of IL-2 received a second course of therapy. Among these patients, two achieved PRs after course 2, and with further therapy, one of these individuals ultimately achieved a CR. Sixty-three patients in the renal cell cancer cohort who had no response to course 1 of IL-2 received a second course of therapy. Among these patients, two achieved PRs and one an MR after course 2, and with additional therapy, one of these three attained a CR. There were eight patients in the melanoma cohort and nine in the renal cell cancer cohort who had no response to courses 1 and 2 of IL-2. These patients went on to receive a third course of IL-2. Interestingly, none of these patients responded to therapy.

This study can be helpful in guiding decisions regarding the administration of high-dose bolus IL-2 in patients with metastatic melanoma or renal cell cancer. Patients with evidence of clinical responses should be treated aggressively, being offered treatment courses until there is disease progression or an inability to tolerate IL-2. In our experience, this generally resulted after the administration of two or three courses of therapy. It is encouraging that, with successive courses of IL-2, additional tumor regression could be observed and that some responses could be converted from partial to complete. This was most evident in the renal cell cancer cohort, among whom eight of 21 patients who had achieved PRs after two courses of therapy were classified as having achieved CRs after a third course of IL-2 (Table 4).

Patients with stable disease after the first course of IL-2 therapy should be considered for a second course of therapy. This recommendation is based on the recognition that, although these patients may be less likely to respond to a second course of IL-2, there are few attractive alternative treatments. After two courses of therapy, patients with stable disease are unlikely to respond to additional therapy and should not be retreated. These guidelines apply only to the high-dose bolus IL-2 regimen used in this study. Definitive data concerning the impact of multiple courses of treatment on the incidence of responses can be derived only from prospective studies in which patients are randomized to receive a predetermined number of treatment courses. To our knowledge, no such studies are in progress or planned.

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Factors Associated With Response to High-Dose Interleukin-2 in Patients With Metastatic Melanoma

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Purpose: The present study attempted to identify characteristics that correlated with clinical response to interleukin (IL)-2 therapy in patients with metastatic melanoma.

Patients and Methods: We retrospectively evaluated laboratory and clinical characteristics of 374 consecutive patients with metastatic melanoma treated with high-dose intravenous bolus IL-2 (720,000 IU/kg) from July 1, 1988, to December 31, 1999, at the Surgery Branch of the National Cancer Institute.

Results: The overall objective response rate was 15.5%. Pretreatment parameters such as patient demographics, laboratory values, and prior therapy did not correlate with response; however, 53.6% of patients with only subcutaneous and/or cutaneous metastases responded, compared with 12.4% of patients with disease at other sites (P_2 = .000001). During therapy, patients who were responders tended to have received more doses during course 1 (16.2 ± 0.3 doses v 14.5 ± 0.2 doses; P_2 = .0095); however, when limited to patients who were able to complete both cycles of course 1, there was no statistically significant difference (P_2 = .27). Responders had a higher maximum lymphocyte count immediately after therapy compared with nonresponders (P_2 = .0026). The development of abnormal thyroid function tests and vitiligo after therapy was associated with response (thyroid-stimulating hormone, P_2 = .01; free T4, P_2 = .0049; vitiligo, $P_2 < 10^{-6}$), although thyroid dysfunction may have been related more to the length of IL-2 therapy than to response.

Conclusion: The presence of metastases only to subcutaneous and/or cutaneous sites, lymphocytosis immediately after treatment, and long-term immunologic side effects, especially vitiligo, were associated with antitumor response to IL-2 therapy.

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THE INCIDENCE OF cutaneous malignant melanoma continues to increase faster than any other cancer in the United States. Approximately 51,000 new cases of invasive malignant melanoma will be diagnosed this year. Approximately one in 74 Americans will develop this malignancy in his or her lifetime.[1] The death rate is also increasing. Nearly 8,000 Americans will die this year from malignant melanoma.[2] Survival is directly related to staging. The 5-year survival rate for those with stage I is more than 95% and decreases significantly to less than 2% for those with stage IV. The median survival time for patients with stage IV disease is approximately 7 months.[3]

The only chemotherapy agent approved by the Food and Drug Administration for the treatment of patients with metastatic melanoma is dacarbazine, which may have a response rate of up to 25%. However, durable responses are extremely unusual, with the majority of patients relapsing within several weeks to months. Treatment with interleukin-2 (IL-2), also approved by the Food and Drug Administration, is associated with a 15% objective response rate. About a third of these patients experience a complete response, the majority of which are durable and probably curative.[4]

IL-2 is a 15-kd glycoprotein produced by helper T-lymphocytes that plays a varied and critical role in immunoregulation. Early experimental studies demonstrating the ability of IL-2 to mediate the regression of established metastases in mice[5] led to clinical trials in patients with metastatic cancer that showed the effectiveness of IL-2 treatment in humans as well.[6,7]

By 1994, the Surgery Branch at the National Cancer Institute (NCI) had established a standard dosing regimen for patients with high-dose IL-2 (720,000 IU/kg intravenously [IV] every 8 hours as tolerated for up to 5 days) and reported a series of 283 patients with metastatic melanoma and renal cell cancer.[8] Melanoma patients had an objective response rate of 17%, with 7% having complete disappearance of assessable metastases; patients with renal cell cancer had a response rate of 20%, with 7% being complete. Follow-up in 1998 showed that over 70% of those complete responders remain ongoing[4]; in fact, no relapses occurred in melanoma patients who maintained a complete response more than 30 months.[9,10] This high-dose bolus IV IL-2 (HD IV IL-2) regimen was approved by the Food and Drug

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Administration in 1998 for the treatment of patients with metastatic melanoma.

Despite these durable responses, a significant factor limiting the use of HD IV IL-2 is the toxicity caused by IL-2. Although the side effects are transient and resolve when IL-2 administration is stopped, they can involve most organ systems. However, HD IV IL-2 can be safely administered, and in a series of patients treated at the NCI since 1987, treatment-related mortality was 0.3%.[11]

Because of these potential toxicities, we attempted in this study to identify characteristics of patients with metastatic melanoma who responded to treatment with HD IV IL-2 alone (in comparison to those who did not respond) which may be useful in identifying more appropriate IL-2 candidates. This article updates a previous publication[12] from this institution that included patients with renal cell cancer and those receiving lower-doses of IL-2, lymphokine-activated killer cells, and IL-2 conjugated to polyethylene glycol, all of which may have affected the prior analysis and were not included in this study.

PATIENTS AND METHODS

Patients

Consecutive patients with assessable metastatic melanoma treated between January 1, 1988, and December 31, 1999, with HD IV bolus IL-2 (720,000 IU/kg every 8 hours as tolerated for up to 15 doses) were included in this study. Patients were enrolled with the intention to complete at least two cycles (one course) of therapy, although some did not complete both cycles because of either toxicity or progressive disease. All patients signed informed consent before protocol enrollment. The institutional review board of the NCI approved all protocols.

Patients were not included if they had any previous exposure to IL-2, if they had received IL-2 conjugated to polyethylene glycol, or received any concurrent cell therapy (lymphokine-activated killer cells, tumor-infiltrating lymphocytes, or dendritic cells), other cytokines (including interferon alfa or tumor necrosis factor), chemotherapy, N^G-monomethyl-L-arginine, monoclonal antibodies, or corticosteroids. At least 4 weeks were required between undergoing any systemic therapy and the first cycle of IL-2 therapy. In the past several years, we started using vaccine therapy directed against melanoma-associated antigens (such as gp100, MART-1, tyrosinase, and TRP1). These vaccines were given as an IV, subcutaneous, or intramuscular injection every 2 to 4 weeks, either before the patient advanced to HD IV IL-2 therapy or in conjunction with HD IV IL-2. No grade 3 or 4 toxicity was noted from the vaccines themselves. Thus these patients who also received HD IV IL-2 were included in this analysis.

IL-2 Therapy

Recombinant IL-2 (provided by Cetus Oncology Division, Chiron Co, Emeryville, CA) was reconstituted by the NCI pharmacy from lyophilized powder in 5% human serum albumin and administered as a 15-minute IV infusion. IL-2 was administered every 8 hours as tolerated up to 15 doses or until the development of a grade 3 or 4 toxicity not easily reversed by supportive therapy, any evidence of neurologic toxicity, or patient refusal. Patients then received another cycle of IL-2 approximately 10 to 20 days after their last doses, although some patients were not able to return for their second cycle because of disease progression.

Response Evaluation

All patients were staged before treatment and subsequently at appropriate evaluation intervals (in general, after two cycles or one course of therapy or 2 months from the initiation of therapy) with appropriate radiologic studies to document their disease. All patients underwent initial computed axial tomography or magnetic resonance imaging of the brain, chest, abdomen, and pelvis as well as radionucleotide bone scans. Plain x-rays or photographs were used as needed to evaluate disease sites. For each patient, the product of the maximum perpendicular diameter of all tumors before and after treatment was compared. An objective partial response was defined as the reduction of $\geq$ 50% of the sum of the products of the maximum perpendicular diameters of all assessable lesions lasting at least 1 month with no new or enlarging tumors. A complete response was defined as the disappearance of all assessable tumor sites lasting at least 1 month. Patients not achieving these criteria were deemed nonresponders.

Statistical Analysis

Univariate analyses were performed to evaluate associations with response. Continuously measured parameters such as laboratory values were compared between responders and nonresponders using the Wilcoxon rank sum test; dichotomous parameters (such as sex or the presence or absence of toxicity) were compared using the χ^2 test or Fisher's exact test. All P values are two-sided (P_2). P_2 values were not adjusted using Bonferroni's correction. Statistical significance was considered only when $P_2 < .01$. A possible noteworthy trend was considered when $P_2 < .05$.

RESULTS

Patient Demographics

Three hundred seventy-four patients were analyzed, consisting of 243 (65%) men and 131 (35%) women, with ages ranging from 16 to 81 years (median, 44 years). Three hundred eighteen patients (85%) had an Eastern Cooperative Oncology Group (ECOG) score of 0; 49 (13%) had an ECOG score of 1; and seven (2%) had an ECOG score of 2. Only 2% of patients were nonwhite.

Response

The overall objective response rate was 15.5% (58 responders of 374 patients). Approximately 5.1% of patients achieved a complete response with disappearance of all assessable metastases, and 10.4% achieved a partial response with reduction of $\geq$ 50% but less than 100%.

Pretreatment Factors Versus Response

Patients' sex, age, race, ECOG score, weight, pretreatment laboratory values (WBC, lymphocyte, and platelet counts, creatinine, bilirubin, and calcium), and prior therapy, such as immunotherapy, interferon alfa, surgery, che-

Table 1. Pretreatment Factors Versus Response

	No. of Patients	Responders No.	Responders %	P_2
Sex				
Male	243	39	16.0	.69
Female	131	19	14.5	
Race				
White	367	58	15.8	.60
Nonwhite	7	0	0.0	
ECOG score				
0	318	54	17.0	.15
1	49	3	6.1	
2	7	1	14.3	
Prior immunotherapy (any except IL-2)				
Yes	192	26	13.5	.28
No	182	32	17.5	
Prior interferon alfa				
Yes	128	17	13.3	.39
No	246	41	16.6	
Prior radiation				
Yes	65	4	6.2	.02*
No	309	54	17.5	
Prior surgery				
Yes	370	57	15.4	.49
No	4	1	25.0	
Prior chemotherapy				
Yes	108	13	12.0	.24
No	266	45	16.9	
Prior hormonal therapy				
Yes	34	5	14.7	.89
No	340	53	15.6	

	Objective Response	Mean ± SEM	Median	P_2
Age, years	Yes	46.3 ± 1.5	47.5	.098
	No	43.8 ± 0.6	44.0	
Weight, kg	Yes	83.7 ± 2.4	50.0	.26
	No	80.4 ± 1.0	45.6	
Baseline lymphocyte, count/μL	Yes	1,450 ± 70	1,447	.15
	No	1,351 ± 32	1,313	
Baseline WBC, count/μL	Yes	6,400 ± 200	6,100	.71
	No	6,600 ± 100	6,300	
Baseline platelet count, × 10^3/mm^3	Yes	230.4 ± 11.7	230.0	.11
	No	258.2 ± 6.5	247.5	

*Odds ratio, 3.23; 95% confidence interval, 1.12 to 12.72.
Abbreviation: ECOG, Eastern Cooperative Oncology Group.

motherapy, or hormonal therapy, were not significantly associated with response (Table 1). There was a trend toward a worse response in those who had prior radiation compared with those to did not (6.2% *v* 17.5%, respectively; P_2 = .02). Although this may suggest some possible immunosuppression from radiation therapy, most likely those who required radiation therapy initially may have had worse initial tumor burden.

Table 2. Sites of Metastases Versus Response

Site of Metastases	No. of Patients	Responders No.	Responders %	P_2
SQ and/or cutaneous alone				
Yes	28	15	53.6	.000001*
No	346	43	12.4	
SQ/cutaneous + LN alone				
Yes	23	5	27.7	.38
No	351	53	15.1	
LN alone				
Yes	29	6	20.7	.42
No	345	52	15.1	
Visceral alone				
Yes	69	8	11.6	.32
No	305	50	16.4	
Visceral + SQ/cutaneous alone				
Yes	61	9	14.8	.86
No	313	49	15.7	
Visceral + LN alone				
Yes	56	5	8.9	.14
No	318	53	16.7	
Bone + any other site(s)				
Yes	30	5	16.7	.80
No	344	53	15.4	
Brain + any other site(s)				
Yes	21	1	0.05	.056
No	353	57	16.1	

*Odds ratio, 8.13; 95% confidence interval, 3.33 to 19.81.
Abbreviations: SQ, subcutaneous; LN, lymph node(s).

The most predictive pretherapy factor for response was the presence of only subcutaneous and/or cutaneous metastases (Table 2). These patients had a response rate of 53.6%, compared with 12.4% in the remaining patients (P_2 = .000001). Of note, a negative trend was noted for patients with brain metastases; only one of these patients had an objective response (P_2 = .056).

In-Treatment Factors Versus Response

Among all 374 patients, responders averaged more doses of IL-2 per course 1 (16.2 ± 0.3; median, 16; range, 10 to 23) than nonresponders (14.5 ± 0.2; median, 15; range, three to 26; P_2 = .0095). However, when limited to those who were able to complete both cycles of course 1 (331 patients), there was no significant difference.

In addition, the development of grade 3 or 4 toxicity (hypotension, tachycardia, arrhythmia, myocarditis or abnormal creatinine kinase, pulmonary insufficiency, oliguria, diarrhea, and neurologic/mental status changes) and the reasons for the cessation of IL-2 dosing (hypotension; arrhythmia; pulmonary insufficiency; neurologic/mental status changes; renal insufficiency; malaise; diarrhea; abnormal creatinine kinase, bilirubin, or plate-

Table 3. Laboratory Values After Starting IL-2 Administration Versus Response

	Objective Response	Mean ± SEM	Median	Range	P_2
Lymphocyte peak, count/μL	Yes	5,878 ± 330	5,464	2,013-12,146	.0026
	No	4,894 ± 141	4,361	588-15,163	
Change in lymphocyte, count/μL*	Yes	4,408 ± 323	3,931	544-10,134	.007
	No	3,539 ± 133	3,028	−1,827-13,705	
Lymphocyte nadir, count/μL	Yes	76 ± 8	60	0-264	.67
	No	93 ± 9	66	0-2,272	
WBC peak, count/μL	Yes	14,100 ± 700	13,000	6,400-31,600	.10
	No	13,100 ± 300	12,200	3,900-33,400	
Platelets peak, × 10^3/mm^3	Yes	465.5 ± 27.8	474.0	0.3-929.0	.10
	No	432.9 ± 12.8	416.0	0.2-1,490.0	
Platelets nadir, × 10^3/mm^3	Yes	66.1 ± 4.9	62.0	0.03-147.0	.053
	No	84.3 ± 3.7	71.0	0.05-574.0	

*Peak lymphocyte count after IL-2 minus pre–IL-2 lymphocyte count.

lets; and patient refusal) were not associated with response.

Most notable, however, was the difference in lymphocytosis between responders and nonresponders (Table 3). The absolute lymphocyte counts were recorded immediately before therapy and daily from the initial administration of IL-2 to the time of discharge. In general, the lymphocyte count peaked 2 to 5 days after cessation of IL-2. Responders had a higher mean maximum lymphocyte count immediately after therapy (by 984/μL compared with nonresponders; $P_2 = .0026$) as well as a higher change in lymphocyte count (maximum value minus pretreatment value), with responders having a greater change (by 869/μL compared with nonresponders; $P_2 = .007$). There was no significant association between response and WBC counts, platelet counts, bilirubin, creatinine, and calcium levels.

Posttreatment Factors Versus Response

In comparing long-term posttreatment parameters, responders were more likely to develop abnormal thyroid-stimulating hormone levels (thyroid-stimulating hormone [TSH]; $P_2 = .01$), abnormal free T4 (FT4) levels ($P_2 = .0049$), and vitiligo ($P_2 < 10^{-6}$) (Table 4). Given the confounding factor that these long-term immunologic side effects could be due to prolonged IL-2 therapy, which would not occur unless the patient continued to respond to IL-2, we then limited our evaluation to the presence of abnormal TSH or FT4 or the presence of vitiligo by day 60 after the start of IL-2 therapy or before the initiation of course 2. Twenty-two patients developed abnormal TSH levels; three were responders, and 19 were nonresponders ($P_2 = .46$). Twenty-seven patients developed abnormal FT4

Table 4. Posttreatment Factors Versus Response and the Incidence of Long-Term Immunologic Side Effects of IL-2 in Responders

	No. of Patients	Responders		Odds Ratio	95% CI	P_2
		No.	%			
TSH						
Normal	153*	15	9.8	2.25	1.16-4.54	.01
Abnormal	219*	43	19.6			
FT4						
Normal	172*	17	9.9	2.35	1.24-4.60	.0049
Abnormal	200*	41	20.5			
Vitiligo						
Present	84	28	33.3	4.33	2.29-8.14	$< 10^{-6}$
Absent	290	30	10.3			

	Abnormal TSH		Abnormal FT4		Vitiligo	
	No.	%	No.	%	No.	%
Responders	43/58	74.1	41/58	70.7	28/58	48.3
Nonresponders	176/314	56.1	159/314	50.6	56/316	17.7

Abbreviation: CI, confidence interval.

*The total number of assessable patients was 372 because two nonresponders did not have TSH/FT4 levels during follow-up.

levels; four of them were responders, and 23 were nonresponders (P_2 = .47). Only one patient (a nonresponder) developed new vitiligo by day 60.

Given that a high percentage of patients developed abnormal TSH (58.9%; 219 of 372 assessable patients) and abnormal FT4 (53.8%; 200 of 372 patients) during and after IL-2 therapy, we attempted to find out whether these abnormalities persisted. Limited by the fact that many nonresponders were not evaluated at long-term follow-up, with at least 6 months after the administration of the last IL-2 dose of course 1, 51.0% (26 of 51 assessable patients) had abnormal TSH, and 49.0% (25 of 51 assessable patients) had abnormal FT4. At least 1 year after course 1, 39.1% (nine of 23 assessable patients) continued to have abnormal TSH, and 52.2% (12 of 23 assessable patients) continued to have abnormal FT4.

DISCUSSION

Several prior studies have attempted to identify predictive factors for IL-2 response. One study reported the negative correlation with serum IL-6 and C-reactive protein levels in patients with renal cell cancer.[13] In a study of 81 patients receiving various IL-2 regimens, increased C-reactive protein levels and the presence of visceral metastases were found to be negatively associated with response.[14]

The current study represents the largest single institution series of patients with metastatic melanoma receiving HD IV IL-2. A previous report from this institution evaluating predictive factors included 112 of these 374 patients.[12]

Overall, the strongest statistical predictor of response was the presence of only subcutaneous and/or cutaneous metastases. This finding has been previously noted from this institution.[12,15]

Prior studies[12,16] have shown an increased response rate in melanoma patients receiving higher total doses of IL-2. When assessable patients were limited to those who were able to receive both cycles of therapy (331 of 374 patients) to correct for the confounding factor that nonresponders were less likely to return for subsequent cycles and those with poor status secondary to tumor burden were less able to complete both cycles, we did not find any significant association between response and the number of IL-2 doses. The number of doses between the two groups showed significant overlap (Table 2).

Rebound lymphocytosis is one of the many hematologic side effects of IL-2.[17] Lymphopenia occurs within minutes of an infusion of IL-2,[18] probably due to margination of lymphocytes. Approximately 24 hours after cessation of IL-2 therapy, a rebound lymphocytosis occurs that persists for 2 to 7 days.[19,20] Although some studies with smaller cohorts did not show an association with lymphocytosis,[20,21] we found a strong positive association between response and lymphocytosis, as did some other reports.[4,22]

Thyroid dysfunction, mainly hypothyroidism, has been found to be common in patients receiving IL-2 therapy. Among initially euthyroid patients, 32% developed hypothyroidism during and 14% after IL-2 therapy.[23] The mechanism seems to be autoimmune, as elevated levels of antithyroglobulin and antithyroid microsomal antibodies have been found.[23,24] Although thyroid dysfunction seemed to be related to response in our initial data, a significant bias exists because patients who respond continue to receive IL-2, which can increase the incidence of abnormal TSH and FT4. When limited to an evaluation checkpoint of less than 60 days or before the initiation of course 2, no significant association existed between thyroid dysfunction and response. In fact, the frequency of thyroid dysfunction has been found to be significantly associated with IL-2 treatment duration.[24,25]

The presence of vitiligo has been reported in patients with metastatic melanoma without any treatment and has been found to be a good prognostic indicator in some patients.[26-28] Since some melanoma-associated antigens (such as MART-1, gp100, and tyrosinase) have been found in normal melanocytes, the incidence of vitiligo suggests that the cellular mechanisms responsible for IL-2 response (activated T lymphocytes) can potentially cross-react with normal tissue. A previous study from this institution evaluating 74 patients with metastatic melanoma found a strong relationship ($P_2 < .005$) between vitiligo and IL-2 response.[29] Interestingly, no patients with renal cell cancer in that study exhibited vitiligo ($P_2 < .0001$), strongly suggesting that vitiligo was due to cross-reactivity, with T cells reacting against one of the melanoma-associated antigens. The current study strengthens this association ($P_2 < 10^{-6}$). Because vitiligo takes time to develop, it is difficult to separate whether there is a real association with response or if vitiligo is purely due to prolonged treatment with IL-2 (which would occur in responders) and the ability to observe responding patients for longer periods of time.

In summary, this single-institution experience with 374 consecutive patients with metastatic melanoma treated with HD IV IL-2 shows that factors strongly related to response include having metastases to only subcutaneous and/or cutaneous sites, lymphocytosis immediately after IL-2 treatment, and the development of vitiligo. Abnormal thyroid function tests may be related to response but the issue is complicated by the fact that continual IL-2 therapy (as would occur in continual responders) increases the incidence of thyroid dysfunction.

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ORIGINAL REPORT

Randomized Phase III Trial of High-Dose Interleukin-2 Versus Subcutaneous Interleukin-2 and Interferon in Patients With Metastatic Renal Cell Carcinoma

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Authors' disclosures of potential conflicts of interest are found at the end of this article.

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ABSTRACT

Purpose
The Cytokine Working Group conducted a randomized phase III trial to determine the value of outpatient interleukin-2 (IL-2) and interferon alfa-2b (IFN) relative to high-dose (HD) IL-2 in patients with metastatic renal cell carcinoma.

Patients and Methods
Patients were stratified for bone and liver metastases, primary tumor in place, and Eastern Cooperative Oncology Group performance status 0 or 1 and then randomly assigned to receive either IL-2 (5 MIU/m^2 subcutaneously every 8 hours for three doses on day 1, then daily 5 days/wk for 4 weeks) and IFN (5 MIU/m^2 subcutaneously three times per week for 4 weeks) every 6 weeks or HD IL-2 (600,000 U/kg/dose intravenously every 8 hours on days 1 through 5 and 15 to 19 [maximum 28 doses]) every 12 weeks.

Results
One hundred ninety-two patients were enrolled between April 1997 and July 2000. Toxicities were as anticipated for these regimens. The response rate was 23.2% (22 of 95 patients) for HD IL-2 versus 9.9% (nine of 91 patients) for IL-2/IFN ($P = .018$). Ten patients receiving HD IL-2 were progression-free at 3 years versus three patients receiving IL-2 and IFN ($P = .082$). The median response durations were 14 and 7 months ($P = .14$), and median survivals were 17.5 and 13 months ($P = .24$). For patients with bone or liver metastases ($P = .001$) or a primary tumor in place ($P = .040$), survival was superior with HD IL-2.

Conclusion
This randomized phase III trial provides additional evidence that HD IL-2 should remain the preferred therapy for selected patients with metastatic renal cell carcinoma.

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INTRODUCTION

In 1992, the United States Food and Drug Administration approved high-dose (HD) bolus interleukin-2 (IL-2; Proleukin; Chiron, Emeryville, CA) for the treatment of patients with metastatic renal cell carcinoma. Approval was based on the finding that IL-2 induced durable responses associated with prolonged disease-free survival in a small percentage of patients.[1,2] However, this regimen was associated with significant toxicity and cost, and consequently, its application has been limited to highly selected patients treated at specialized centers.[3,4] Several investigators have evaluated regimens containing lower doses of IL-2 in an attempt to decrease toxicity.[5-7] Attempts were also made to improve treatment efficacy by adding interferon alfa-2b (IFN; Intron A; Schering Plough Corporation, Kenilworth, NJ) and then fluorouracil to

low-dose IL-2 regimens. These regimens were reported to produce response rates and survival comparable to those reported for HD IL-2 with much less acute toxicity.[8-12]

In an effort to confirm and extend these results, the Cytokine Working Group (CWG) conducted a series of phase II trials that evaluated HD bolus IL-2 alone, intravenous (IV) IL-2 and IFN, outpatient subcutaneous IL-2 and IFN, and subcutaneous IL-2 and IFN alternating with fluorouracil/IFN in patients with metastatic renal cell carcinoma.[13-15] All patients on these studies had met the same eligibility criteria. The response rates (range, 11% to 17%) and median survivals (range, 15 to 20 months) were similar in these studies, although acute toxicity was less severe in the outpatient regimens. The addition of IV IFN to HD IL-2 and fluorouracil to outpatient IL-2 and IFN did not seem to improve efficacy but did increase toxicity. The median response duration and 3-year progression-free survival seemed to be longest with HD IL-2; however, because these studies were not randomized, patient selection bias could have influenced the results.

This phase II experience encouraged the CWG to formally investigate whether lower-dose IL-2 regimens were able to produce durable responses at a rate similar to HD IL-2 before accepting such regimens as standard therapy.

Therefore, a randomized phase III trial was initiated to compare HD IV bolus IL-2 with outpatient subcutaneous IL-2 and IFN in patients with advanced renal cancer. On the basis of the results of earlier CWG phase II studies, 3-year progression-free survival was chosen as the primary study end point.

PATIENTS AND METHODS

Patient Selection

Eligible patients were required to have histologically confirmed bidimensionally measurable and clearly progressive metastatic renal cancer; an Eastern Cooperative Oncology Group performance status of 0 or 1; adequate organ function, with normal hematologic parameters; serum creatinine $\leq$ 1.5 mg/dL or calculated creatinine clearance greater than 60 mL/min; forced expiratory volume in 1 second greater than 2.0 L/sec or 75% of predicted value; no evidence of congestive heart failure, serious cardiac arrhythmias, symptoms of coronary artery disease, or ischemia on a cardiac stress test; negative serologic testing for human immunodeficiency virus type I antibody and hepatitis B surface antigen; no contraindications to the use of pressor agents; no evidence of active infection requiring antibiotic therapy; and no medical condition requiring corticosteroids. Four weeks were required to elapse since prior therapy; patients who had received prior treatment with either IL-2 or IFN and those with brain metastases, seizure disorders, organ allografts, history of another malignancy, or concurrent corticosteroid therapy were ineligible. The human investigational research committee at each institution approved the protocol at all institutions and voluntary written informed consent was obtained from each patient.

Treatment Plan

Outpatient subcutaneous IL-2 and IFN. On treatment day 1, patients received a subcutaneous IL-2 loading dose of 5×10^6 U/m^2 every 8 hours for three doses. This was followed by a 5×10^6 U/m^2 dose via subcutaneous injection, one dose per day on treatment days 2, 3, 4, and 5 (week 1), and then daily 5 days per week for the remaining 3 weeks as outpatients. During the first 4 weeks of treatment, patients also received subcutaneous IFN 5×10^6 U/m^2/dose thrice weekly. Cycles were repeated every 6 weeks. One cycle consisted of 4 weeks of treatment followed by 2 weeks of rest. Up to 2 weeks of additional rest were allowed for the resolution of adverse events. A maximum of six 6-week cycles were given.

Patients were premedicated with acetaminophen 500 to 650 mg orally every 4 hours (total 2,600 mg to 3,000 mg/d). Oral nonsteroidal anti-inflammatory drugs were administered to patients whose fever was unresponsive to acetaminophen. Opioid analgesia (meperidine 25 to 50 mg orally) was given for severe rigors. Patients were evaluated for tumor response after cycles 1, 2, 4, and 6. Patients with disease progression at any time were ineligible for further treatment. All patients were treated for at least two 6-week cycles unless progressive disease or unacceptable toxicity was encountered. To be eligible for more than two cycles, patients had to have at least stable disease, with some evidence of tumor regression or an objective response, and had to meet baseline eligibility criteria for organ function.

High-dose IV IL-2. Patients received IL-2 600,000 U/kg/dose (Chiron) IV every 8 hours for 5 days (maximum of 14 doses) beginning on day 1 and again on day 15. One cycle consisted of 5 days of treatment, 9 days of rest, 5 more days of treatment, and 9 weeks of rest. A treatment delay of up to 4 weeks was allowed for resolution of side effects between cycles. Patients were eligible to receive a maximum of three cycles of treatment.

Patients underwent placement of a central venous catheter before each course of therapy and received antibiotic prophylaxis with ciprofloxacin 250 mg orally bid on days 1 to 10 and 15 to 24 of each cycle. All antihypertensive therapy was discontinued at least 24 hours before initiating each cycle of IL-2. Patients also received acetaminophen (650 mg orally every 4 hours) and indomethacin (25 mg every 6 hours) to reduce febrile reactions, ranitidine (150 mg) or famotidine (20 mg) orally every 12 hours for prophylaxis of gastrointestinal bleeding, hydroxyzine hydrochloride (25 to 50 mg orally every 6 hours) or diphenhydramine (25 mg orally every hours) for pruritus, meperidine (25 to 50 mg orally every 6 hours) for chills and rigors, an antidiarrheal agent, antiemetics, anxiolytics, diuretics, and vasopressors as needed.

Patients were evaluated for response during week 6 and 12 of the first cycle. To be eligible for more than one cycle of treatment, patients must have had at least stable disease with evidence of some minor tumor regression or objective response and had to meet baseline eligibility criteria for organ function.

Dose Modification and Toxicity Monitoring

Toxicity was evaluated using the National Cancer Institute (NCI) Common Toxicity Criteria version 2.0.

Dose modification for toxicity: IL-2 and IFN. Dose-limiting toxicity (DLT) was defined as grade 3 to 4 toxicity with the exception of cardiac and neurologic toxicity ($\geq$ grade 2) and hematologic and liver toxicity (grade 4). If a patient developed a DLT during weeks 1 to 4 of any cycle, both IL-2 and IFN were held until recovery took place (ie, the DLT improved to grade 1 or less) and then reinstituted with no change in dose. If a DLT recurred, doses for both drugs were reduced by 40% thereafter. If a DLT recurred

at the lower dose, treatment was stopped and the patient was taken off treatment.

Dose modification for toxicity: HD IL-2. Treatment with HD IL-2 was modified by withholding doses of IL-2 rather than continuing therapy at a reduced dose. Doses of IL-2 were withheld for hypotension refractory to fluids and pressors or requiring unacceptably high pressor doses, anuria for more than 24 hours and unresponsive to fluid replacement and low-dose dopamine, respiratory distress requiring more than 4 L of oxygen to maintain O_2 saturation greater than 95%, confusion, sustained ventricular tachycardia or any sign or symptom of myocardial ischemia or myocarditis, metabolic acidosis with HCO_3 less than 18 despite attempts to correct with IV HCO_3; atrial fibrillation, documented systemic infection, or any other serious toxicity that was not controlled at time of next dose.

Response Assessment

Standard response criteria were used. Complete response (CR) was defined as the complete absence of all clinical evidence of malignant disease for at least two determinations 4 weeks apart. Partial response (PR) required a greater than 50% decrease in the sum of the products of the perpendicular diameters of all measurable lesions for at least two measurements at least 4 weeks apart. Minor response was defined as less than 50% but more than 25% reduction, but was in fact considered stable disease. Stable disease was defined as including minor response, no change, or less than 25% increase in disease and no new disease. Clinically relevant stable disease had to exceed 6 months. Progressive disease was defined as a greater than 25% increase in the sum of the products of perpendicular diameters of all lesions or the appearance of any new lesion. All patients who achieved a CR or PR had their computed tomography scans audited by independent radiologists to confirm their response and response duration.

Statistical Methods

The primary objective of this phase III study was to determine whether HD IL-2 was superior to outpatient subcutaneous IL-2 and IFN in terms of 3-year progression-free survival. Based on prior studies, it was assumed that the percentage of patients who would remain progression-free at 3 years was 10% for those receiving HD IL-2% and 2% in the IL-2 and IFN arm. The sample size was calculated to detect a difference in 3-year progression-free survival of 8% between the arms with 90% power. We presumed that 5% of enrolled patients would be found to be ineligible. The accrual of 174 patients was required to achieve this power.

After the study was underway, data began to emerge that suggested that patients with non–clear-cell primary tumors did not respond to biologic therapy. The accrual goal was then increased by 10% to permit subset analysis of only clear-cell patients at a later date, thus bringing the final total to 193 patients. Registration and randomization of eligible patients was performed at Beth Israel Deaconess Medical Center. Patients were randomly assigned to one of the two treatment arms in equal proportions using a stratified permuted block randomization. Before randomization, patients were stratified based on Eastern Cooperative Oncology Group performance status (0 or 1), liver or bone metastasis (yes or no), and primary tumor in place (yes or no). Additional prognostic criteria, as described by Motzer et al, [16] were collected from patient records after study completion.

Baseline continuous variables were summarized as median and range and compared between treatment arms using the Wilcoxon rank sum test. Binary baseline and response variables were compared between arms using Fisher's exact test; exact binomial CIs were reported. Three-year progression-free survival and 3-year durable CR were observed for all patients and are analyzed as binary end points. Time-to-event variables were summarized using Kaplan-Meier curves. Response duration was defined from date of documented tumor response to date of documented progressive disease or was censored at date of last follow-up visit; a log-rank test was used to compare treatment arms. Survival end points were defined from date of randomization to date of documented progressive disease (for progression-free survival) or death from any cause or were censored at date of last follow-up visit. Cox proportional hazards regression models were used to estimate hazard ratios and calculate log-rank tests (ie, score test) comparing treatment arms. The model among all patients was stratified by the three randomization strata. For the models by randomization variables, each was stratified for the other two randomization variables. The assumption of proportionality between treatment arms was assessed by plots of log of the cumulative hazard versus time and by testing for an interaction term of treatment arm with time in the model. For patients without liver or bone metastases, the assumption seemed violated, and time-varying hazard ratios were calculated for selected clinically relevant time points. Two-sided P values were reported for all analyses. The statistical analysis used SAS 8.2 (SAS Institute Inc, Cary, NC) and StatXact-5 (Cytel Software Corp, Cambridge, MA).

RESULTS

Patient Characteristics

One hundred ninety-three patients were enrolled at 10 participating institutions between April 1997 and July 2000. One patient withdrew consent before treatment and could not be followed-up for any study end point. Ninety-six patients were assigned to each treatment. All patients met the eligibility criteria, but six refused therapy after randomization (five randomly assigned to IL-2 and IFN; one randomly assigned to HD IL-2). These patients were not evaluated for response or progression-free survival but were followed for overall survival. Median duration of follow-up was 4.9 years (range, 3.4 to 6.0 years). The characteristics of patients on this study are listed in Table 1.

Treatment arms were evenly balanced for the stratification criteria and were reasonably well balanced for prior therapy, sex, age, and the Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria.[16] Forty-five percent of all patients had liver or bone metastases, 31% had their primary tumors in place, and 87% were intermediate or poor risk by the (MSKCC) prognostic criteria.

Treatment

Treatment information is listed in Table 2. During the first 12 weeks of therapy, patients received most of the planned doses of IL-2 and IFN and 68% of the planned doses of HD IL-2.

Toxicity

The incidence of grade 3 and 4 toxicity in cycle 1 of treatment is listed in Table 3. Grade 3 and 4 toxicities were

Table 1. Patient Characteristics

	% of Patients		
	IL-2 and IFN (n = 96)	HD IL-2 (n = 96)	P*
ECOG PS			.88
0	61	59	
1	39	41	
Liver or bone metastases			.88
No	56	54	
Yes	44	46	
Primary tumor			.88
Out	68	70	
In	32	30	
Sex			.21
Male	64	73	
Female	36	27	
Age, years			.28
Median	56	53	
Range	21-75	25-74	
Prior systemic therapy	3	2	
Motzer prognostic criteria[1]			.25
Good	14	12	
Intermediate	73	82	
Intermediate/poor	3	3	
Poor	10	3	

Abbreviations: IL-2, interleukin-2; IFN, interferon alfa-2b; HD, high-dose; ECOG, Eastern Cooperative Oncology Group.
*Because of inadequate data, six patients could not be classified at all (n = 5, IL-2 and IFN; n = 1, HD IL-2), and six patients may have been intermediate or poor risk.

more common with HD IL-2. In general, the side effects with both treatment regimens were typical of our prior published experience with these regimens.[4,13-15] However, one patient receiving IL-2 and IFN developed acute renal failure while on treatment and required permanent hemodialysis. In addition, two treatment-related deaths were noted, one on each treatment arm. A 44-year-old male patient died during cycle 1, week 4 of IL-2 and IFN of acute respiratory distress syndrome and progressive lung metastases. A 60-year-old male died during cycle 1, week 1 of HD IL-2 as a result of complications from capillary leak syndrome.

Table 2. Planned Treatments Received During Initial 12 Weeks

	IL-2 and IFN, 6-Week Cycles (%)		HD IL-2, 12-Week Cycles (%)	
	Mean	±SE	Mean	±SE
Cycle No. 1				
IL-2	93	16	68	19
IFN	92	18	NA	
Cycle No. 2				
IL-2	95	12		
IFN	95	11		

Abbreviations: IL-2, interleukin-2; IFN, interferon alfa-2b; HD, high-dose; NA, not applicable.

Table 3. Grade 3 and 4 Toxicities in Cycle 1

	IL-2 and IFN (n = 91)		HD IL-2 (n = 95)	
	No. of Patients	%	No. of Patients	%
Constitutional	13	14.3	3	3.2
Hypotension	1	1.1	54	56.8
Gastrointestinal	13	14.3	9	9.5
Hematologic	0	0	13	13.7
Neurologic	3	3.3	14	14.7
Cardiac	0	0	8	8.4
Pulmonary	1	1.1	13	13.7
Renal/electrolytes	3	3.3	13	13.7
Psychiatric	1	1.1	0	0
Hepatic	2	2.2	11	11.6
Infection	0	0	3	3.2

Abbreviations: IL-2, interleukin-2; IFN, interferon alfa-2b; HD, high-dose.

Response Data

Tumor response data by treatment arm are listed in Table 4. The overall response rate to HD IL-2 was 23.2% (95% CI, 15.1% to 32.9%), compared with 9.9% (95% CI, 4.6% to 18.0%) with IL-2 and IFN ($P = .018$). There were eight complete responses (8.4%) with HD IL-2, compared with only three responses (3.3%) on the IL-2 and IFN arm ($P = .214$).

Tumor response data by treatment arm for each of the three stratification criteria are listed in Table 5. Statistically significant differences in response rate favoring the HD IL-2 regimen were seen for patients with liver or bone metastasis ($P = .008$) and primary tumor in place ($P = .024$).

The median response duration for HD IL-2 was 24 months, compared with 15 months for IL-2 and IFN ($P = .180$; Fig 1). The median progression-free survival was 3.1 months for each treatment arm (Fig 2). Ten patients (nine responders and one patient with stable disease) receiving HD IL-2 remained progression-free at 3 years,

Table 4. Summary of Tumor Response Data

	IL-2 and IFN (n = 91)		HD IL-2 (n = 95)		
	No. of Patients	%	No. of Patients	%	P*
Overall response	9	9.9	22	23.2	.018
CR	3	3.3	8	8.4	.214
PR	6	6.6	14	14.7	
Durable 3-year CR	0	0	7	7.4	.014

Abbreviations: IL-2, interleukin-2; IFN, interferon alfa-2b; HD, high-dose; CR, complete response; PR, partial response.
*By Fisher's exact test.

Table 5. Summary of Tumor Response by Randomization Strata

	IL-2 and IFN (n = 91)		HD IL-2 (n = 95)		
	%	No. of Patients	%	No. of Patients	P*
ECOG PS					
0	9.1	5/55	23.2	13/56	.070
1	11.1	4/36	23.1	9/39	.227
Liver or bone metastases					
No	15.4	8/52	23.5	12/51	.329
Yes	2.6	1/39	22.7	10/44	.008
Primary tumor					
Out	14.1	9/64	24.2	16/66	.183
In	0	0/27	20.7	6/29	.024

Abbreviations: IL-2, interleukin-2; IFN, interferon alfa-2b; HD, high-dose; ECOG, Eastern Cooperative Oncology Group; PS, performance status.
*By Fisher's exact test.

compared with three patients (two responders and one patient with stable disease) who received IL-2 and IFN ($P = .082$). Nine of 22 patients who responded to HD IL-2 remain progression-free at 38 to 63 months, whereas only one of nine patients who responded to IL-2 and IFN (a PR) remain progression-free (51 months). There are seven ongoing CRs on HD IL-2 and none on IL-2 and IFN ($P = .014$).

Survival Data

One hundred ninety-two patients were followed up for survival. Survival by treatment arm, stratification subset, and MSKCC prognostic criteria is shown in Table 6. Median survival from time on-study was 13 months for patients assigned to IL-2 and IFN therapy and 17 months for those assigned to HD IL-2. This trend in survival benefit favoring HD IL-2 was not statistically significant ($P = .211$; Fig 3).

A statistically significant survival benefit was noted for patients with liver or bone metastases ($P = .001$) and for patients with primary tumors in place ($P = .040$) with HD IL-2 therapy (Fig 4A through 4F).

Broadly similar results were found when the analysis was limited to the 165 patients with clear-cell histology (77 patients receiving IL-2 and IFN; 88 patients receiving HD IL-2). There was still a significant response difference for patients with liver or bone metastases ($P = .036$), a trend in the number of patients progression-free at 3 years ($P = .067$), and a survival benefit for patients with liver or bone metastases ($P = .002$) or primary tumors in place ($P = .034$) all favoring HD IL-2.

DISCUSSION

Although HD IL-2 produces durable high-quality responses in a small percentage of patients with metastatic renal carcinoma, its toxicity and cost have limited its

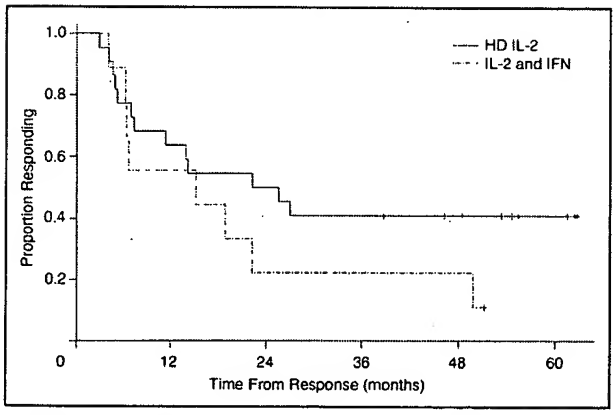

Fig 1. Duration of response to therapy by treatment arm among 31 patients who responded to high-dose interleukin-2 (HD IL-2; n = 22) or IL-2 and interferon alfa-2b (IFN; n = 9). $P = .180$ by log-rank test.

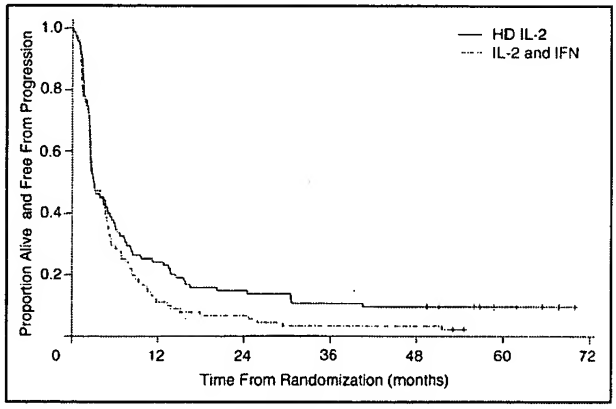

Fig 2. Progression-free survival by treatment arm among 186 patients receiving high-dose interleukin-2 (HD IL-2; n = 95) or receiving IL-2 and interferon alfa-2b (IFN; n = 91). Ten patients receiving HD IL-2 remained progression-free at 3 years compared with three patients who received IL-2 and IFN ($P = .082$ by Fisher's exact test).

Table 6. Summary of Overall Survival, All Patients and by Randomization Strata

	No. of Deaths/Patients	Median Survival (months) IL-2 and IFN (n = 96)	Median Survival (months) HD IL-2 (n = 96)	Hazard Ratio* All Patients	Hazard Ratio* HD	Hazard Ratio* IL-2 and IFN	P
All patients	159/192	13.0	17.1	0.81	0.59	1.13	.211
ECOG PS							
0	92/116	19.3	23.5	0.87	0.57	1.32	.509
1	67/76	8.5	9.1	0.74	0.44	1.23	.241
Liver or bone metastases							
Not	86/106	22.1	21.1				—
6 months				2.18	1.08	4.39	
12 months				1.36	0.88	2.12	
24 months				0.91	0.53	1.57	
Yes	73/86	8.0	14.7	0.46	0.28	0.75	.001
Primary tumor							
Out	107/132	18.1	20.7	0.97	0.66	1.43	.878
In	52/60	8.2	12.4	0.54	0.29	0.98	.040
MSKCC criteria							
Good	15/24	26.9	30.9		—		—
Intermediate	121/144	13.9	16.8	0.77	0.52	1.12	.171
Intermediate/poor	5/6	8.2	3.0		—		—
Poor	12/12	3.9	1.6		—		—

Abbreviations: IL-2, interleukin-2; IFN, interferon alfa-2b; HD, high-dose; ECOG, Eastern Cooperative Oncology Group; PS, performance status; MSKCC, Memorial Sloan-Kettering Cancer Center.

*The models for all patients and for MSKCC criteria were stratified for three randomization strata; otherwise models were stratified for the other two randomization strata. *P* values are stratified log-rank tests. Only intermediate MSKCC category was analyzed because of small numbers in other categories.

†For patients without liver or bone metastases, the hazard ratio was not proportional over time and is thus presented as a function of time at three clinically relevant time points. Initially better survival was seen among patients receiving IL-2 + IFN relative to patients receiving HD IL-2, but by 12 months the difference is no longer statistically significant.

application to selected patients treated at specialized centers.[1-4] Lower dose IL-2–based regimens have been reported to produce similar response and survival rates with less toxicity, leading to their widespread use in this patient population.[5-12] Phase II studies conducted by the CWG have suggested that lowering the dose of IL-2 might result in fewer durable responses.[13-15] Before accepting low-dose IL-2 and IFN as standard therapy for metastatic renal cell carcinoma, we designed a randomized phase III study to compare the relative value of HD IL-2 and low-dose IL-2 and IFN.

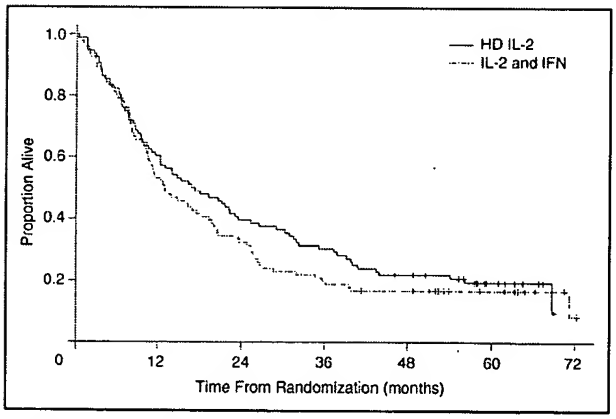

Fig 3. Overall survival among 192 patients randomly assigned to receive high-dose interleukin-2 (HD IL-2; n = 96) or IL-2 and interferon alfa-2b (IFN; n = 96). *P* = .211 by log-rank test.

In this study, HD IL-2 produced a statistically significant improvement in response rate (23.2% *v* 9.9% *P* = .018) compared with IL-2 and IFN. The response quality, as reflected by the CR rate (8% *v* 3%), durable CR rate (7.4% *v* 0%), and response duration (median 24 *v* 15 months) also favored HD IL-2 treatment, although only durable CR rate was statistically significant. HD IL-2 did not have a significant impact on median progression-free survival or median overall survival. Given that IL-2–based therapy for metastatic renal cell carcinoma benefits a minority of patients, we did not expect to see significant differences in these survival end points. However, this study confirmed the observation from prior CWG phase II studies, showing a trend in the number of patients free of disease progression at 3 years (10 *v* three patients; *P* = .082) favoring HD IL-2 therapy.

Patients with performance status ≥ 1, primary tumor in place, or liver or bone metastases have been reported to be less likely to respond to IL-2–based therapy.[17] Consequently, we stratified study patients for these variables. Of note, patients with liver or bone metastases and patients treated with their primary tumor in place had significantly

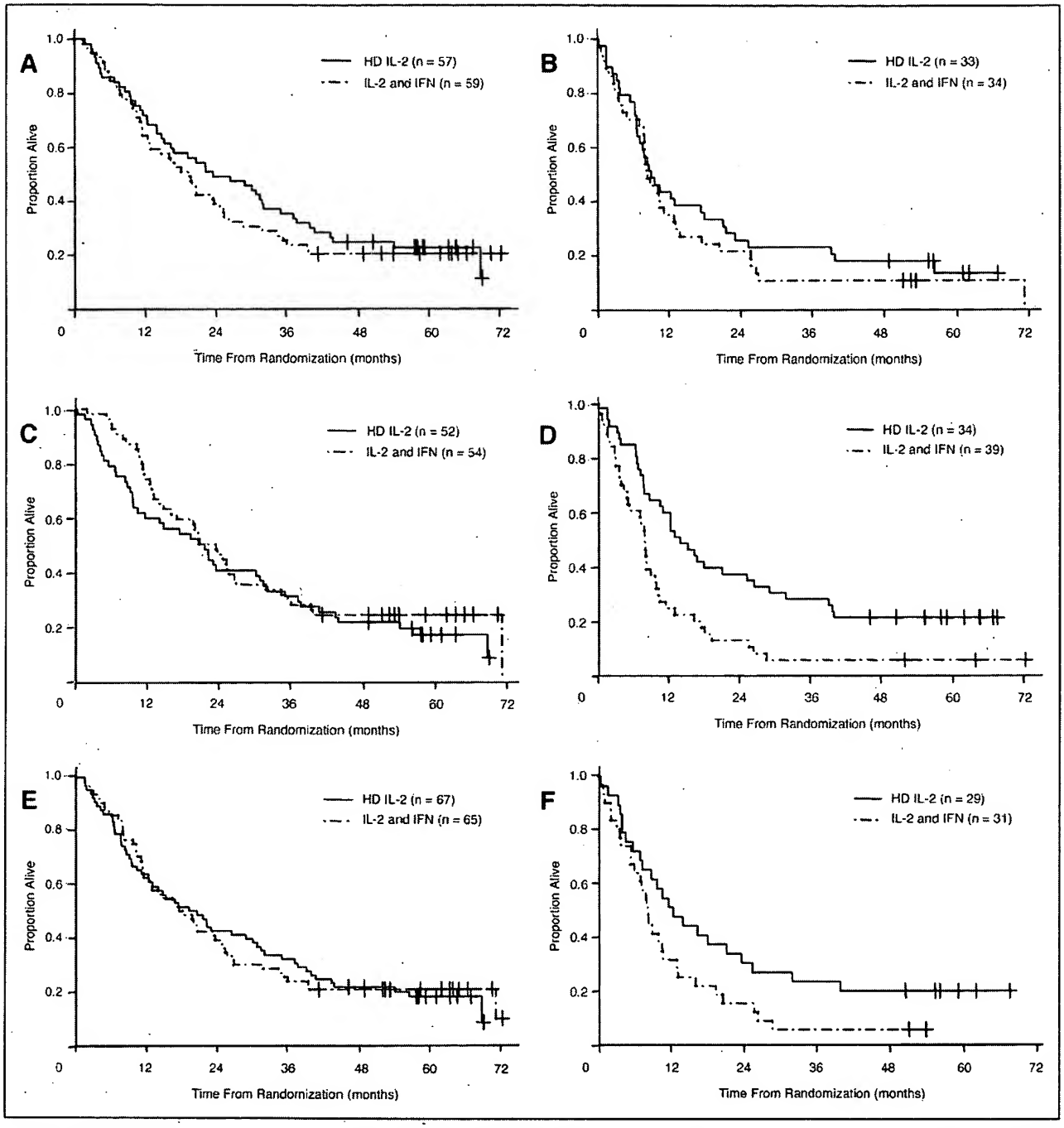

Fig 4. Overall survival among 192 patients randomly assigned to receive high-dose interleukin-2 (HD IL-2) or IL-2 and interferon alfa-2b (IFN). (a) Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0; (b) ECOG PS of 1; (c) without liver or bone metastases; (d) with liver or bone metastases; (e) primary out; (f) primary in place.

improved response rates and survival with HD IL-2 relative to lower-dose IL-2 and IFN. The current study represents the first time a survival advantage has ever been shown for any stratified subset of patients receiving IL-2–based therapy. However, the results of the subset analyses should be seen as hypothesis generating and will need to be confirmed in future trials.

Although the response rate for patients receiving HD IL-2 remained relatively constant across all prognostic variables (21% to 24%), IL-2 and IFN was essentially inactive in

patients who had liver or bone metastases or their primary tumor in place. The explanation for this unanticipated result is not readily apparent. It is possible that higher serum or tissue IL-2 levels are needed either to overcome the immune suppression associated with greater tumor burden or to activate T cells in sites of disease other than lung and soft tissue. Consequently, it seems that patients with liver or bone metastases or unresected primaries represent a group of patients who seem to require a more intensive IL-2 regimen to achieve clinical benefit, whereas the impact of dose is less critical in patients with resected primaries and tumor confined to lung.

In contrast to earlier CWG phase II trials, the response rate to HD IL-2 in this study was significantly higher than the response rate with IL-2 and IFN. Considered in the light of this trial, the previously observed similarities in response rates might have been the consequence of less rigorous auditing of the responses in the prior IL-2 and IFN studies than was performed for the HD IL-2 data set that was prepared for United States Food and Drug Administration submission. Although the median response duration seen for patients on the HD IL-2 arm of this study was shorter than in the prior CWG phase II HD IL-2 trial (54 *v* 24 months), the fact that the plateau on the response duration curve falls just below the 50% mark probably accounts for this difference. As expected, HD IL-2 produced more acute toxicity than outpatient IL-2 and IFN. However, holding therapy rapidly reversed most toxicity, and treatment-related mortality occurred in only one patient on each arm.

Other investigators have previously studied the relative value of IL-2–based regimens in patients with metastatic renal cancer. In 1998, the French Immunotherapy Group reported on a large, phase III randomized study that compared inpatient continuous-infusion IL-2 alone with either IFN alone or the combination of IL-2 and IFN.[10] They concluded that the combination of IL-2 and IFN was superior in terms of response rate and 1-year progression-free survival as compared with monotherapy with either agent. This study used an IL-2 regimen that was less intensive than bolus HD IL-2, which may explain the low level of antitumor activity in the IL-2 alone arm (8% response rate). In addition, although IL-2 and IFN produced a superior response rate, this did not translate into more durable responses or an improvement in median or overall survival. In 2003, NCI Surgery Branch investigators reported results of a randomized phase III trial that compared the efficacy and toxicity of HD IV IL-2 to a lower-dose IV regimen (10% of high dose) using an otherwise identical administration schedule.[18] In that study, patients who received HD IL-2 had a significantly higher response rate (21% *v* 13%; $P = .048$) and were more likely to have durable responses than those who received the lower-dose IV regimen. However, there were no significant differences in overall survival between the two groups. Despite the more acute toxicity with the high-dose regimen, quality-of-life assessments showed no differences between the two treatment arms. In an overlapping three-arm study, a third group of patients were randomly assigned to receive a low-dose outpatient subcutaneous IL-2 regimen. In this three-arm comparison, the response rates were 21% for HD IL-2, 11% for lower-dose IV IL-2%, and 10% for subcutaneous IL-2. Once again there were no significant survival differences. In this study, the vast majority of patients had an excellent performance status and had undergone prior nephrectomy, and relatively few had liver or bone metastases, making subset analyses difficult to perform. In the CWG study, most of the trend in survival difference favoring HD IL-2 was attributable to the patient populations with liver or bone metastases and primary tumor in place, perhaps explaining why the survival difference was less pronounced in the NCI Surgery Branch trial. Taking these three randomized studies into consideration, one can conclude that HD IL-2 is superior to both lower doses of IL-2 or IL-2 and IFN in terms of response rates and response quality.

This CWG phase III randomized trial provides additional evidence that HD IL-2 should remain the preferred therapy for selected patients with access to such treatment. Furthermore, it suggests that patients with bone or liver metastases or a primary tumor in place may receive little benefit from a lower-dose IL-2 regimen. Given the toxicity and expense associated with HD IL-2 therapy, and the still-low overall response rate, these results suggest that better criteria for selecting patients for HD IL-2 therapy are necessary. Although some progress has been made in this regard, even with the best selection criteria, the majority of patients will not respond to IL-2 therapy.[19-22] Consequently, new treatment options that focus on important targets (eg, angiogenesis and signal transduction) will still be necessary for those with unfavorable selection features or who experience disease progression after IL-2–based therapy.

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Chiron; Kim A. Margolin, Chiron, Schering; Michael B. Atkins, Chiron. Performed contract work within the last 2 years: Lawrence E. Flaherty, Chiron, Schering. Received more than $2,000 per year from a company for either of the last 2 years: Joseph I. Clark, Chiron, Schering; Lawrence E. Flaherty, Chiron, Schering; Geoffrey R. Weiss, Chiron, Schering; Jeffrey A. Sosman, Chiron; Marc S. Ernstoff, Chiron, Schering; Kim A. Margolin, Chiron, Schering; Jared A. Gollob, Chiron, Schering; Janice P. Dutcher, Chiron; and Michael B. Atkins, Chiron.

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ERRATA

In the January 1, 2005, Erratum (J Clin Oncol 23:248, 2005), there was an omission.

The erratum was printed without a DOI number. The DOI number for this erratum is 10.1200/JCO.2005.12.910. This omission occurred in the printed issue only, and the DOI is readily available and searchable on www.jco.org.

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The results section of the abstract mistakenly states that the median response durations were 14 and 7 months ($P = .14$) for high-dose IL-2 and outpatient IL-2 and IFN, respectively. The correct median response durations are 24 and 15 months ($P = .18$).

The Authors' Disclosures of Potential Conflicts of Interest section contained an omission. David F. McDermott received more than $2,000 per year from Chiron for either of the last 2 years.

The online version of the article was corrected in departure from the print.

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Low Dose Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and Interleukin-2 (IL-2) Are Well Tolerated Following Autologous Stem Cell Transplant (ASCT) in Patients (pts) with Hematologic-Malignancies (Meeting abstract).

Sub-category: Bone Marrow Transplantation/Cytokines
Category: Bone Marrow Transplantation/Cytokines
Meeting: 1999 ASCO Annual Meeting

Abstract No: 205

Author(s): D Abramovich, B Bolwell, B Pohlman, S Andresen, D Neely, K Wise, M Kalaycio

Abstract: GM-CSF upregulates IL-2 receptors on lymphocytes. IL-2 has known activity against leukemia, lymphoma and multiple myeloma. We hypothesized that the combination of GM-CSF and IL-2 might be more efficacious than IL-2 alone, and if given after ASCT for these malignancies, might improve freedom from relapse. We therefore designed a pilot study to assess the feasibility and tolerability of low dose GM-CSF and IL-2 administered after ASCT. 13 pts, 6 males, 7 females, mean age of 47 (34--59) with various hematologic malignancies (non-Hodgkin's lymphoma (7), Hodgkin's disease [HD](3), multiple myeloma (1) and acute myelogenous leukemia [AML](2), treated with busulfan, cyclophosphamide and etoposide with ASCT were enrolled after recovery from ASCT. All had an ANC >1500/ml, a platelet count >35K/mg, ECOG performance status <2, normal hepatic, pulmonary and cardiac functions and no active disease or infections at enrollment. All were treated with GM-CSF 125mcg/m[Superscript 2]/d SQ and IL-2 1million U/m [Superscript 2]/d SQ M--F for 8 weeks starting within 8 weeks following discharge from ASCT. Pts were assessed by physical exam and chest x-ray at 4 and 8 weeks in our clinic. CBC's and chemistries were checked weekly and pts were contacted weekly by phone to assess for toxicity. Pts were followed after completion of the GM-CSF/IL-2 at the discretion of the treating physician. Pts exhibited the following grade 1 toxicities: Fatigue (56), myalgia/bone pain (5), fever/chills (5), headache/dizziness (4), nausea/vomiting (5), diarrhea (3), rash (7), shortness of breath (2), pedal edema (2), leukopenia (2), chest pain (1). All pts developed mild and self limiting irritation and induration at sites of injection of IL-2. Pts exhibited the following grade 2 toxicities: Rash (1), angioedema (1), herpes zoster (3). The 2 pts exhibiting leukopenia were AML pts in relapse. 11/13 pts completed therapy. 2 pts terminated therapy early due to relapsed AML. No pt stopped therapy because of toxicity. No grade 3 or 4 toxicities were encountered. All toxicities were reversible. There was no adverse impact on engraftment, although 5 pts developed an absolute eosinophilia of >1500/ml during GM-CSF/IL-2 which resolved following completion of GM-CSF/IL-2. There have been 3 relapses to date, 2 AML pts and 1 HD pt who possibly relapsed prior to initiating GM-CSF/IL-2. Conclusion: IL-2 and GM-CSF in the doses and schedule used in this study are well tolerated as adjunctive therapy to ASCT for hematologic malignancies. Low dose GM-CSF/IL-2 warrants further evaluation in prospective studies as a means of improving outcomes following ASCT.

Other Abstracts in this Sub-Category

1. **Lack of Appropriate Caregivers Limits Utilization of Outpatient Autologous Stem Cell Transplantation (ASCT) (Meeting abstract).**
   Meeting: 1999 ASCO Annual Meeting Abstract No: 156 First Author: P Frey
   Category: Bone Marrow Transplantation/Cytokines
2. **A Parallel, Randomized, Filgrastim Controlled and Open-Label Dose Ranging Study of Peg G-CSF (Ro 25-8315) in Breast Cancer Patients with Metastatic Disease or Locoregional Recurrence Treated with A Combination of Adriamycin, Cyclophosphamide and Mesna (Meeting abstract).**
   Meeting: 1999 ASCO Annual Meeting Abstract No: 157 First Author: P Viens
   Category: Bone Marrow Transplantation/Cytokines
3. **Adenovirus Rather Than BK Virus Is the Important Pathogen of Hemorrhagic Cystitis After Bone Marrow Transplantation. (Meeting abstract).**
   Meeting: 1999 ASCO Annual Meeting Abstract No: 158 First Author: H Akiyama
   Category: Bone Marrow Transplantation/Cytokines

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Abstracts by D Abramovich

1. **Low Dose Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and Interleukin-2 (IL-2) Are Well Tolerated Following Autologous Stem Cell Transplant (ASCT) in Patients (pts) with Hematologic-Malignancies (Meeting abstract).**
   Meeting: 1999 ASCO Annual Meeting Abstract No: 205 First Author: D Abramovich
   Category: Bone Marrow Transplantation/Cytokines
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1. **Effect of a Vascular Endothelial Growth Factor (VEGF) Inhibitory Treatment on the Folliculogenesis and Ovarian Apoptosis in Gonadotropin-Treated Prepubertal Rats.**
   Biol Reprod,
   Vol , No (6/16/2006): pp.
   PMID: 16775226 [PubMed - in process]
2. **Effect of gonadotropin-releasing hormone agonist and antagonist on proliferation and apoptosis of human luteinized granulosa cells.**
   Fertil Steril, United States
   Vol 85, No 4 (4/4/2006): pp. 1064-7
   PMID: 16580402 [PubMed - in process]
3. **Interrelations between ceruloplasmin and fe status during human pregnancy.**
   Biol Trace Elem Res, United States
   Vol 98, No 1 (Apr, 2004): pp. 1-12
   PMID: 15051895 [PubMed - in process]
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Pilot Study of Low-Dose Interleukin-11 in Patients With Bone Marrow Failure

By Razelle Kurzrock, Jorge Cortes, Deborah A. Thomas, Sima Jeha, Susan Pilat, and Moshe Talpaz

Purpose: **Interleukin-11 (IL-11) is a thrombopoietic cytokine that attenuates postchemotherapy thrombocytopenia at doses of 50 μg/kg/d subcutaneously. Very little is known about the activity of IL-11 in patients with bone marrow failure states.**

Patients and Methods: **Our preliminary experience with IL-11 at doses of 50 μg/kg/d suggested that patients with bone marrow failure developed significant peripheral and pulmonary edema after the prolonged dosing necessary for treating these conditions. We, therefore, initiated a study of low-dose IL-11 (starting dose, 10 μg/kg/d).**

Results: **Sixteen patients were assessable for response. Six patients had diploid cytogenetics; the others had a variety of chromosomal abnormalities. Six (38%) of 16 patients showed a platelet response to IL-11, and two had a multilineage response (to IL-11 alone, n = 1; to IL-11 plus G-CSF and erythropoietin, n = 1). The median increase in peak platelet counts was 95 × 10^9/L above baseline in the responders (range, increase of 55 × 10^9/L to 130 × 10^9/L above baseline). Responders included five of 11 patients with myelodysplasia and one of four patients with aplastic anemia. Response durations were 12, 13, 14+, 25, 30, and 30+ weeks. Side effects of IL-11 were mild (peripheral edema, n = 7; conjunctival injection, n = 7; myalgia, n = 1; all grade 1). Seven patients had no side effects.**

Conclusion: **Our pilot study suggests that administration of low-dose IL-11 (10 μg/kg/d) can raise platelet counts without significant toxicity in selected thrombocytopenic patients with bone marrow failure.**

J Clin Oncol 19:4165-4172. © 2001 by American Society of Clinical Oncology.

PATIENTS WITH myelodysplasia (MDS) and other bone marrow failure states (aplastic anemia, graft failure, and so on) suffer from low platelet counts and an increased risk of serious hemorrhage.[1,2] Currently, therapeutic options in these patients are limited to platelet transfusion support. However, platelets are short-lived, and, therefore, any benefits from platelet transfusions generally last 3 days or less. Platelet transfusions are also not without side effects. For instance, such transfusions can transmit bacterial and viral infections, and, furthermore, repeated administration of platelets often results in immune refractoriness.[3,4]

To date, growth factors (granulocyte colony-stimulating factor [G-CSF] and granulocyte-macrophage colony-stimulating factor [GM-CSF]) developed for clinical use have been successful in increasing WBC counts in patients suffering from both primary and secondary neutropenia.[5,6] In some patients with bone marrow failure, erythropoietin can increase hemoglobin and decrease RBC transfusion requirements.[5,7,8] However, little in the way of salutary effects on platelet counts have been reported. Interleukin-11 (IL-11), a molecule that stimulates megakaryocytopoiesis in rodents and monkeys, has been found to attenuate thrombocytopenia and reduce the need for platelet transfusions after myelosuppressive chemotherapy in patients with nonmyeloid malignancies who are at high risk for severe thrombocytopenia.[9-12] The effects of this molecule in bone marrow failure states has not, however, been previously investigated.

The usual dose of IL-11 administered after chemotherapy is 50 μg/kg/d.[11] The main side effects at this dose are mild anemia and reversible arthralgia, dyspnea, edema, and tachycardia. However, tolerance is good for most patients. Our initial experience with the use of this molecule in patients who experience bone marrow failure suggested that doses between 25 and 50 μg/kg/d resulted in significant peripheral and pulmonary edema, probably because these patients require prolonged therapy (unpublished data). Therefore, we initiated a pilot study of IL-11 at low doses (10 μg/kg/d subcutaneously [SC]). Preliminary results suggest that low-dose IL-11 is biologically active and well tolerated in patients with bone marrow failure.

PATIENTS AND METHODS

Patients with bone marrow failure as a result of MDS, aplastic anemia, graft failure, or postchemotherapy aplasia were eligible for the protocol. Diagnosis was made on the basis of a review of bone marrow aspirate and biopsy and karyotype analysis. Patients could not have received chemotherapy for at least 2 months or have evidence of progressive cancer (other than worsening MDS). Patients must not have

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0732-183X/01/1921-4165/$20.00

received antithymocyte globulin for a least 3 months and not have received corticosteroids, danazol, or cyclosporine for at least 4 weeks. Other eligibility criteria included a platelet count ≤ 50 × 10^9/L. Patients with known allergies to *Escherichia coli*, active congestive heart failure, or documented myeloid leukemia were excluded. All patients signed informed consent in keeping with our internal review board policies.

Treatment Plan

Patients received at least two courses of IL-11. Each course consisted of 2 weeks of daily IL-11 (10 μg/kg/d SC), followed by a 2-week rest period. After the first two courses (8 weeks), patients who showed any evidence of response could continue receiving maintenance therapy. The length of the courses versus the rest period and the dose of IL-11 could be individualized during the maintenance period in accordance with patient response and side effects. In particular, dosing would be adjusted to maintain platelet counts between 150 and 450 × 10^9/L. Baseline evaluation included a complete blood cell count with differential and reticulocyte count, an ECG, and liver and kidney function tests. A bone marrow aspirate and biopsy with cytogenetic analysis was performed within 1 month before therapy. During therapy, patients were monitored with a complete blood cell count and differential and reticulocyte count three times per week for the first 6 weeks and then at least weekly. Liver and kidney function tests were repeated at least every 2 weeks, and bone marrow aspirate and biopsy were repeated at 4- to 8-week intervals.

Response Criteria

Platelet response was denoted as doubling of platelets, with platelet counts increasing to levels of more than 50 × 10^9/L (for patients with baseline platelet counts between 20 and 50 × 10^9/L) or tripling of platelets and counts increasing to levels of more than 20 × 10^9/L (for patients with baseline platelet counts ≤ 20 × 10^9/L). Baseline platelet and neutrophil counts and hemoglobin were the median of the three untransfused counts available within the 2 weeks before starting therapy. After therapy, transfused platelet counts were not considered in the evaluation of response. Patients were not administered transfusions if platelet counts were more than 10 × 10^9/L. By definition, patients with counts below this level had to become transfusion-independent (and untransfused platelet counts had to increase to more than 20 × 10^9/L) to be considered responders. Patient responses had to last at least 4 weeks while they were on therapy.

RBCs were transfused for hemoglobin ≤ 8 g/dL. Patients were considered to have an RBC response if there was an increase in hemoglobin of 2 g/dL above baseline accompanied by attainment of transfusion independence.

Neutrophil increases were considered responses if there was a tripling of neutrophils and an increase to more than 0.5 × 10^3/μL (if baseline neutrophil count was ≤ 0.5 × 10^3/μL) or a doubling of neutrophils and an increase to more than 1.0 × 10^3/μL (if baseline neutrophil counts were between 0.5 and 1.0 × 10^3/μL).

RESULTS

To date, 20 patients have been registered on trial. Sixteen patients are assessable for response. Four patients were nonassessable because they were registered in error (n = 2), noncompliant (n = 1) or received concurrent thalidomide (n = 1). All but the two patients registered in error were assessable for toxicity (n = 18).

Table 1. Patient Characteristics

		No. of Patients
Patients registered		20
Patients assessable for toxicity		18
Patients assessable for response		16
Reasons patients not assessable		
Registered in error		2
Noncompliant		1
Concurrent thalidomide		1
Age, years		
Median	58	
Range	5-84	
Sex		
Male		14
Female		4
Diagnosis		
RA		5
RARS		1
RAEB		5
AA		4
BMF, after auto-BMT		1
Karyotype		
Diploid		8
Insufficient metaphases		1
Monosomy 7		2
Trisomy 8		2
Monosomy 5		1
11q−		1
20q−		1
13q−		1
Trisomy 15		1
−Y		1
Baseline platelet count, × 10^9/L		
Median	12	
Range	1.0-48	

Abbreviations: AA, aplastic anemia; BMF, bone marrow failure.

Patient Characteristics

The diagnoses of the patients included refractory anemia (RA) (n = 7), refractory anemia with ringed sideroblasts (RARS) (n = 1), refractory anemia with excess blasts (RAEB) (n = 5), severe aplastic anemia (n = 4), and bone marrow failure after autologous bone marrow transplantation (n = 1). Fourteen men and four women were treated. The median age of the patients was 58 years (range, 5 to 84 years) (Table 1). Six patients with MDS had received no prior therapy. The other patients had received one to four prior therapies (Table 2). Eight patients had diploid cytogenetics, and the other patients had a variety of chromosomal abnormalities (Table 1). The median baseline platelet count was 12 × 10^9/L (range, 1 to 48 × 10^9/L).

Responses

Six (38%) of 16 patients assessable for response showed increases in platelets (Table 3). The diagnoses of the

Table 2. Prior Therapy

Prior Therapy	No. of Patients
Antithymocyte globulin	10
Cyclosporine	9
G-CSF	5
Stem-cell factor	4
Topotecan/cytarabine	2
Allogeneic BMT	1
Amifostine	1
None	6

Abbreviation: BMT, bone marrow transplantation.

responders included RA (n = 1), RARS (n = 1), RAEB (n = 3), and aplastic anemia (n = 1). Two patients who responded had diploid cytogenetics, one had insufficient metaphases, and the other had chromosomal abnormalities, including monosomy 5 and 7, trisomy 8, and deletion of the long arm of chromosome 11 (Table 3). Response duration was 12, 13, 14+, 25, 30, and 30+ weeks.

Case no. 1. Patient no. 1 was a 66-year-old man with a diagnosis of RA of 14 months' duration. Karyotype was 46, XY, del(20q13), 11q−. Prior therapy with amifostine was unsuccessful. The initial platelet count was 22 × 10^9/L; hemoglobin, 9.4 g/dL; and absolute neutrophil count, 1.2 × $10^3/\mu$L. The patient showed an increase in platelet counts with IL-11 administration (Fig 1); peak platelet levels were 77 × 10^9/L. The platelet response lasted 15 weeks. Bone marrow biopsy showed an increase in cellularity from 40% (baseline) to 70% during treatment. There was no change in the percentage of blasts or degree of dysplasia. Forty-six megakaryocytes per 10 high-power fields were seen both before and during therapy. (In normal individuals, 20 to 30 megakaryocytes are seen per 10 high-power fields. A high-power field denotes ×400 magnification.) Megakaryocytes were dysplastic. At the time of loss of response, bone marrow megakaryocytes showed a decrease in number to 35 per high-power field. There was no evidence of disease progression.

Table 3. Response to Low-Dose IL-11

	No. of Patients
Patients assessable for response	16
Responders (%)	6 (38)
Diagnosis of responders/total no. of patients	
RA	1/5
RARS	1/1
RAEB	3/5
AA	1/4
Karyotype of responders	
−5, −7, +8	1
t(3;12), 13q−	1
11q−	1
Insufficient metaphases	1
Diploid	2

NOTE. Response durations of the six patients were 12, 13, 14+, 25, 30, and 30+ weeks.

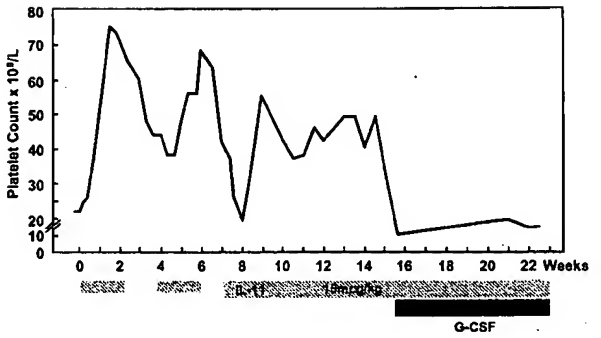

Fig 1. Platelet response of patient no. 1. Courses of IL-11, as well as erythropoietin (EPO) and G-CSF, are depicted in the bars below the graph. IL-11 was initiated at a dose of 10 μg/kg/d and later increased to 15 μg/kg/d.

Case no. 2. Patient no. 2 was a 66-year-old man with RARS of 13 months' duration. Karyotype showed insufficient metaphases. He had received no prior therapy for MDS but had been treated with a fludarabine-based regimen for previous chronic lymphocytic leukemia. His initial platelet count was 48 × 10^9/L, and his absolute neutrophil count was 0.9 × $10^3/\mu$L. He was RBC transfusion–dependent. He showed a platelet response that lasted 14+ weeks (Fig 2); the peak platelet count was 180 × 10^9/L. Bone marrow biopsies showed a slight increase in cellularity during therapy (baseline cellularity, 30%; posttherapy cellularity, 50%). Bone marrow megakaryocytes increased from 21 per 10 high-power fields (baseline) to 62 per 10 high-power fields during treatment. Bone marrow dysplasia did not change significantly.

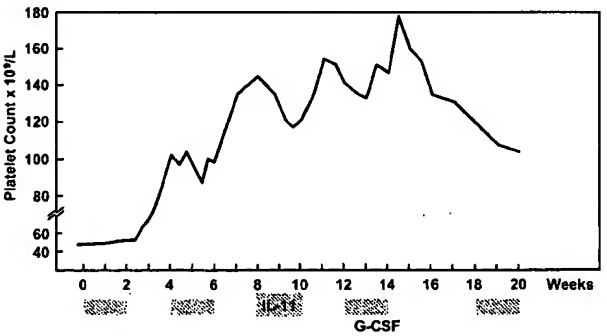

Fig 2. Platelet response of patient no. 2. Courses of IL-11 as well as G-CSF are depicted in the bars below the graph. IL-11 was administered at a dose of 10 μg/kg/d.

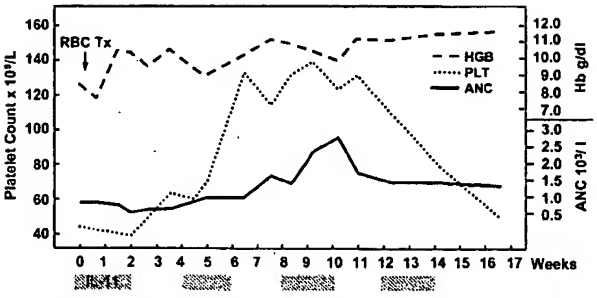

Fig 3. Platelet (PLT) response of patient no. 3. Courses of IL-11 are depicted in the bars below graph. No other growth factors were administered. IL-11 dose was 10 μg/kg/d. Because this patient had a multilineage response, hemoglobin (Hb, HGB) and absolute neutrophil (ANC) responses are depicted. Arrows represent transfusions (Tx).

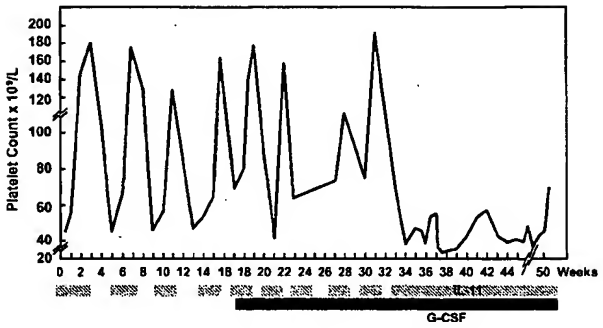

Fig 4. Platelet response of patient no. 4. Courses of IL-11 as well as of EPO and G-CSF are depicted in the bars below the graph. IL-11 dose was 10 μg/kg/d.

Case no. 3. Patient no. 3 was a 56-year-old man with RAEB of 1 month's duration. Karyotype was 46, XY, t(3;12)(p10;p10), del(13q). He had a history of small cleaved lymphoma and had received an autologous transplant 5 years before development of MDS. Baseline blood counts included a platelet count of 44 × 10^9/L and an absolute neutrophil count of 0.9 × 10^3/μL. He was RBC transfusion–dependent. Surprisingly, he had a trilineage response to IL-11. No other growth factor was administered. Peak platelet counts were over 130 × 10^9/L. Hemoglobin increased to 11.5 g/dL, and the absolute neutrophil count increased to 2.5 × 10^3/μL. The response lasted 12 weeks (Fig 3). During therapy, bone marrow biopsy cellularity showed no significant changes from the baseline of 25%. At the time of peak platelet response, bone marrow blasts decreased from 17% (baseline) to 3%. At the time of loss of response, bone marrow blasts increased to 29%. However, 2 weeks later, they decreased back to baseline values without further therapy. Bone marrow megakaryocytes increased from 4 per 10 high-power fields (baseline) to 9 per 10 high-power fields during treatment. There were no significant changes in the degree of dysplasia.

Case no. 4. Patient no. 4 was a 72-year-old man with RAEB of 15 months' duration. Karyotype was diploid. He had received only supportive therapy. Baseline blood counts included a platelet count of 45 × 10^9/L and an absolute neutrophil count of 0.8 × 10^3/μL. He was RBC transfusion–dependent. He had a platelet response to IL-11; peak platelet counts were over 180 × 10^9/L. The response lasted 30 weeks (Fig 4). Bone marrow biopsy cellularity increased from 50% at baseline to 90% during therapy. Neither bone marrow blasts nor the degree of dysplasia changed significantly. Bone marrow megakaryocytes increased from 75 per 10 high-power fields (baseline) to 126 per 10 high-power fields (after therapy).

Case no. 5. Patient no. 5 was a 78-year-old man with RAEB of 3 months' duration. Karyotype showed multiple abnormalities, including monosomy 5 and 7 and trisomy 8. He had received only supportive therapy. Baseline blood counts included a platelet count of 36 × 10^9/L and an absolute neutrophil count of 0.9 × 10^3/μL. He was RBC transfusion–dependent. He had a platelet response to IL-11; peak platelet counts were approximately 90 × 10^9/L. The response lasted 25 weeks (Fig 5). Bone marrow biopsy cellularity remained at approximately 50% to 60% throughout therapy. Neither bone marrow blasts nor the degree of dysplasia changed significantly. Bone marrow megakaryocytes did not change from baseline levels of approximately 26 per 10 high-power fields.

Case no. 6. Patient no. 6 was a 58-year-old woman with severe aplastic anemia of 3 years' duration. Karyotype was diploid. Prior therapy included antithymocyte globulin and cyclosporine, as well as stem-cell factor and G-CSF. Baseline blood counts included a platelet count of 1.0 × 10^9/L and an absolute neutrophil count of 0.6 × 10^3/μL (while receiving G-CSF). She was RBC transfusion–dependent. She had pre-

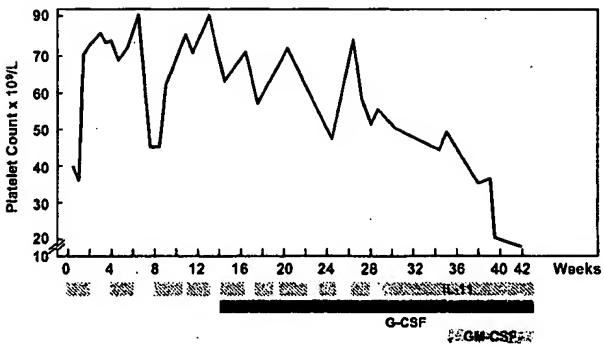

Fig 5. Platelet response of patient no. 5. Courses of IL-11 as well as of EPO, G-CSF, and GM-CSF are depicted in the bars below the graph. IL-11 dose was 10 μg/kg/d.

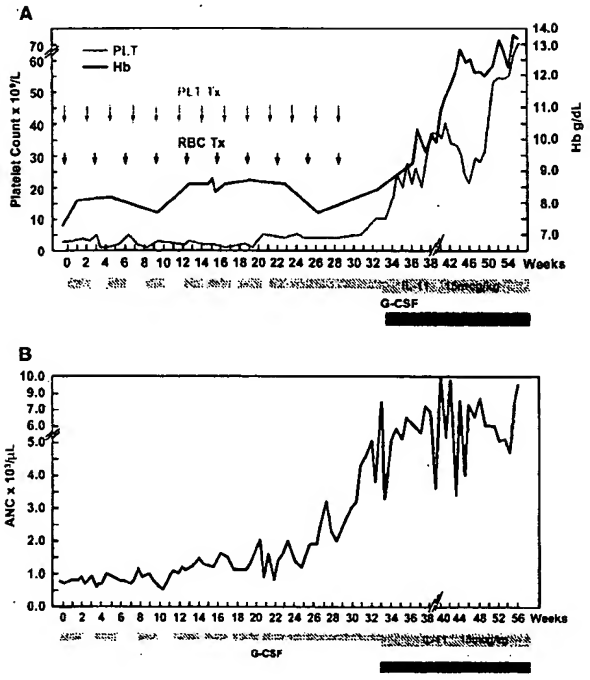

Fig 6. Platelet response of patient no. 6. Bars below graphs depict courses of IL-11, EPO, and G-CSF. Initial IL-11 dose was 10 μg/kg/d; later increased to 15 μg/kg/d. Hemoglobin (A) and ANC (B) response are shown. Arrows represent transfusions.

viously received G-CSF together with stem-cell factor for close to 1 year without a major response in any lineage (including neutrophils). During IL-11 therapy, G-CSF (300 μg/d SC) was continued. All three lineages began to increase after approximately 20 weeks on therapy (at which time the rest period between courses had been discontinued). At 34 weeks, the IL-11 dose was increased from 10 to 15 μg/kg/d SC, and erythropoietin at a dose of 10,000 units SC (three times per week) was added. At this point, blood counts began to increase more rapidly. Peak platelet counts were over 100×10^9/L. Hemoglobin increased to 13.5 g/dL, and absolute neutrophil counts increased to $9.0 \times 10^3/\mu L$. Response duration was 30+ weeks (Fig 6). Bone marrow biopsy cellularity increased from 5% at baseline to 60% during therapy. Neither bone marrow blasts nor the degree of dysplasia changed significantly. Bone marrow megakaryocytes increased from 2 per 10 high-power fields to 11 per 10 high-power fields.

Side Effects

Patient tolerance of low-dose IL-11 was excellent. Seven patients developed peripheral edema, seven showed conjunctival injection, and one complained of myalgias. These toxicities were mild (grade 1). Two patients were given furosemide (20 mg by mouth daily) for mild peripheral edema. Seven patients had no side effects.

DISCUSSION

Bone marrow failure states include a variety of conditions, such as MDS, aplastic anemia, and iatrogenic (chemotherapy-induced) prolonged pancytopenias. In general, G-CSF and GM-CSF can successfully increase neutrophil counts in MDS patients.[5,6,13-31] For some time, the interest in myeloid growth factors was tempered by the concern that their usage led to early transformation. Controlled studies with G-CSF and GM-CSF have refuted this notion.[14-16,18,21,23] However, less than 20% of MDS patients will have a salutary response to erythropoietin,[7,32-42] and platelet responses after growth factor therapy are reported only anecdotally.

Although MDS is a preleukemic state, most patients suffer from and often succumb to the sequelae of cytopenias, without overt progression to leukemia.[1] Management of MDS has included the use of differentiating agents and chemotherapy. Many of these trials have been unrewarding or have yielded excessive toxicity, and a positive impact on survival has not been demonstrated. Aplastic anemia is treated most successfully with bone marrow transplantation or immunosuppression with antithymocyte globulin and cyclosporine.[2] However, for patients who lack sibling donors and do not respond to immunosuppression, there are few options.[43] Graft failure complicates a significant minority of bone marrow transplants. As with other bone marrow failure states, G-CSF and GM-CSF can improve neutrophil counts, but no molecule has proven efficacious at increasing platelet counts. Therefore, for all these cytopenic states, there has been an ongoing interest in the potential beneficial effects of platelet growth factors.

IL-11 is a thrombopoietic cytokine that promotes the growth of hematopoietic stem cells and megakaryocytic progenitors and induces megakaryocyte differentiation, which results in increased platelet counts in animal models of compromised hematopoiesis and in cancer patients after chemotherapy.[9-12] IL-11 administered to mice undergoing bone marrow transplantation after total-body irradiation stimulates platelet and neutrophil recovery.[10] When stem-cell factor is also administered, increases in all three lineages without toxicity is observed. In humans, several studies have demonstrated that IL-11 attenuates chemotherapy-induced thrombocytopenia.[11,44] Currently, IL-11 is the only molecule approved in the United States for ameliorating thrombocytopenia.

The current trial represents the first study of IL-11 for patients in bone marrow failure states. The approved dose of IL-11 postchemotherapy is 50 μg/kg/d SC. In general,

this dose is reasonably well tolerated when administered for the short periods (approximately 7 days) needed after chemotherapy. However, our preliminary experience suggested that the prolonged dosing required in patients with bone marrow failure resulted in significant fluid accumulation when 25 to 50 μg/kg was administered daily (unpublished data). Therefore, we initiated a pilot trial of low-dose IL-11 (10 μg/kg/d). The major objectives of this trial were to ascertain whether these low doses of IL-11 were biologically active and tolerable. Six (38%) of 16 patients showed significant increases in platelet counts (Figs 1 to 6). In most of these patients, platelets increased with the first 2-week course of IL-11. In patients no. 1 and 4, abrupt decreases in platelet counts were seen in the 2-week rest period between courses (Figs 1 and 4). In four patients, significant increases in bone marrow megakaryocytes accompanied the platelet responses. Interestingly, one patient (case no. 3, Fig 3) showed a multilineage response to IL-11 given without any other concomitant growth factor. This is not totally surprising, because IL-11 can influence primitive hematopoietic cell development.[45] One patient (case no. 6) with severe aplastic anemia (initial platelet count, 1.0×10^9/L) showed a multilineage response when IL-11 was combined with G-CSF and erythropoietin. This patient was unusual in that, previous to IL-11 therapy, she had remained severely neutropenic while on G-CSF. Furthermore, the time to response was approximately 20 weeks. We have noted a similar delay in time to response when severe aplastic anemia patients were treated with other growth factors, eg, IL-3/GM-CSF combinations or stem-cell factor.[46,47] In fact, the median time to initial response in our aplastic anemia patients receiving stem-cell factor was 4 months.[47] Alternatively, it may have been a change in the schedule or dose of IL-11 administration that was critical to response. Improvement in platelet counts started when the 2 weeks on/2 weeks off schedule of IL-11 was replaced by continuous administration (Fig 5). The multilineage response was observed several weeks later, when the dose of IL-11 was increased from 10 to 15 μg/kg/d and erythropoietin was added. Bone marrow megakaryocytes and cellularity also increased significantly in this patient.

Several patients eventually ceased to respond. Bone marrow follow-up showed that end of platelet response was not accompanied by other indications of progressive disease, such as transformation to leukemia. Future studies are planned to monitor for development of neutralizing IL-11 antibodies.

Several other thrombopoietic molecules have also been administered to patients with bone marrow failure, with variable results. Thrombopoietin was administered to patients with graft failure without significant therapeutic benefit, albeit in a study that allowed only one to five doses of this molecule monthly.[48] IL-3 has shown limited thrombopoietic activity in patients with aplastic anemia or MDS,[13,49] although multilineage responses have been seen when IL-3 is combined with GM-CSF.[46] Stem-cell factor has also demonstrated multilineage responses in some patients with aplastic anemia.[47] Finally, IL-6 has been reported to have thrombopoietic activity in MDS, albeit with significant toxicity.[50-52] To date, none of the above molecules have demonstrated benefits leading to their approval for any clinical indication in the United States, although some of these molecules (eg, stem-cell factor) have been approved in other countries for indications such as stem-cell mobilization.

Taken together, the data in the literature as well as our pilot study suggest that several cytokines are able to increase platelet counts in subsets of patients experiencing bone marrow failure. Some of these patients may have multilineage responses, especially if combinations of growth factors are used. Our current study demonstrates that IL-11 is biologically active in bone marrow failure states at considerably lower doses than previously used after chemotherapy. At these doses, this molecule is capable of increasing platelet counts in a subset of patients with bone marrow failure without significant toxicity. Because some of the responses were short-lived and occurred in patients with only mild thrombocytopenia, the clinical significance of this biologic activity remains to be ascertained in patients who are transfusion-dependent. Further studies are needed to explore a variety of issues: (1) other schedules of administration (eg, alternate day, high-dose once weekly, and others), (2) combinations of IL-11 with other growth factors or with other treatments, (3) biologic or phenotypic characteristics that correlate with response, (4) mechanisms of response and loss thereof, (5) importance of duration of therapy, especially in patients with severe thrombocytopenia, and (6) overall impact on larger numbers of patients who are platelet transfusion–dependent.

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Hyperthermic Isolated Limb Perfusion With Low-Dose Tumor Necrosis Factor-α and Melphalan for Bulky In-Transit Melanoma Metastases

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Background: Melphalan (L-PAM) hyperthermic isolated limb perfusion (HILP) is currently considered the standard treatment for patients with in-transit metastases from cutaneous melanoma. We here report on the results of L-PAM and low-dose tumor necrosis factor (TNF)α HILP in patients with bulky disease.

Methods: Twenty patients underwent TNFα (1 mg) and L-PAM (10 mg/L) HILP. Perfusion was performed for 90 minutes, and systemic leakage was strictly monitored. Locoregional toxicity was evaluated according to Wieberdink's criteria, whereas tumor response was evaluated with physical examination and ultrasound scan with or without fine-needle aspiration of any suspected recurrence.

Results: In all cases, systemic leakage was <5%. No postoperative deaths occurred, and locoregional toxicity was mild (grade 1 or 2) in 95% of patients. A complete tumor response was obtained in 14 patients (70%), and partial responses were obtained in 5 patients (25%). After a median follow-up of 18 months, six patients are alive and disease free, seven are alive with local or distant recurrence or both, and seven have died of disease.

Conclusions: Low-dose TNFα HILP can achieve tumor responses comparable with those reported with higher doses of cytokine. Moreover, this drug regimen is associated with acceptable local toxicity, carries a smaller risk of systemic toxicity, and incurs lower costs.

Key Words: Melanoma—Isolated limb perfusion—TNFα—Melphalan.

In-transit disease occurs in an estimated 2% to 10% of all cutaneous melanoma patients,[1] and the lower limb is the main location in approximately 70% of cases.[2] Surgical resection is indicated when lesions are small and limited in number, and amputation should be considered only if limb function is severely impaired or if hygienic conditions are poor.

When disease extent is a contraindication to surgical excision, hyperthermic isolated limb perfusion (HILP) should be taken into consideration, because amputation does not provide any advantage in terms of disease-free survival and because the activity of systemic chemotherapy against local disease control is low.[3] HILP is a well-established locoregional procedure that can deliver high-dose cytostatics to a limb with multiple in-transit melanoma lesions.[4] This technique is quite sophisticated and requires accurate monitoring of systemic leakage and limb temperature to avoid major systemic and local side effects. Since its first clinical application, with a reported complete response (CR) rate of approximately 50%, melphalan (L-PAM) has been used as the referral drug.[3,4] Other cytostatics, such as nitrogen mustards, dacarbazine, actinomycin D, and cisplatin, have shown no therapeutic advantage over L-PAM in terms of either response duration or response rates.[3,4]

Since the early 1990s, recombinant human tumor necrosis factor (TNF)α and L-PAM have been used for HILP, with impressive CR rates of up to 90% in several published series.[3,4] However, TNFα administration has potentially lethal side effects if significant systemic leak-

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age occurs during HILP. Particular care must therefore be taken to monitor drug leakage when HILP is performed.[5] Despite the large body of reports on TNFα-based HILP for the treatment of melanoma metastases,[4,6] it is not yet clear whether this regimen improves the tumor response rate, because no definitive data are available on a homogeneous population of melanoma patients. Two prospective randomized clinical trials have been undertaken in the United States and Europe that challenge L-PAM versus L-PAM plus TNFα. In the US trial, the regimen was also combined with interferon-gamma (IFNγ). The European trial was terminated early because of slow recruitment, and no significant difference in tumor CR rate was found between the two study groups.[7] The US study showed that the addition of TNFα and IFNγ improved tumor response rates, especially in the subset of patients with bulky disease.[8] However, because the cytokine was used at the dose of 3 to 4 mg, the optimal TNFα dose in HILP has not yet been clearly established.

Some investigators[9] have suggested that low-dose (.5–1 mg) TNFα might be as active as higher doses. We performed a phase I and II study with L-PAM and escalating dosages of TNFα under hyperthermic conditions (40.5°C–41.5°C) and found similar tumor response rates but fewer complications in patients treated with .5 to 1.6 mg of TNFα than in patients treated with higher dosages.[10] We therefore started to routinely treat patients with recurrent or bulky in-transit melanoma metastases with 1 mg of TNFα plus L-PAM HILP. In this article, we report on and discuss the results of our clinical series.

PATIENTS AND METHODS

Patient Selection

After a routine preoperative clinical work-up and staging, patients were considered eligible if they had histologically confirmed bulky disease confined to a limb and no evidence of distant metastasis and if their clinical condition was satisfactory. The disease was considered bulky if there were more than 15 metastatic lesions or when 1 or more tumor nodules had a diameter >3 cm. Patients who had gross recurrence after previous L-PAM–only based HILP were also included in the study. HILP was considered feasible if the tumor had not extended to the root of the affected limb. All patients gave their signed informed consent before undergoing HILP.

Perfusion Technique and Postoperative Care

HILP was performed under general anesthesia according to a well-established technique.[11–13] Briefly, the main limb vessels were exposed at the root of the limb, dissected free, and encircled with tourniquets. Major tributaries were dissected to identify potential sites of leakage from the perfusion circuit. Moreover, a tourniquet was secured at the root of the limb. Indwelling temperature probes were inserted throughout the limb and connected to a recorder. To monitor the leakage from the perfusion circuit to the systemic circulation, a gamma counter was connected to a rate meter and strip chart recorder and positioned over the heart. After systemic heparinization (200 UI/kg), the main vessels were cannulated and connected to the perfusion circuit, which was already primed with Ringer's lactate. Arterial flow rates were set at 40 to 80 mL/min per kilogram of limb weight. Perfusate was oxygenated and heated to 42°C in a water bath (arterial line Po_2 was >500 mm Hg). A period of 10 to 20 minutes was required for a muscle temperature of 39°C to 40°C (plateau phase) to be reached. Leakage monitoring was performed according to the technique described by Casara et al.[14] by using ^{99m}Tc-albumin in the perfusion circuit. If no significant leakage was recorded, 1 mg of TNF was bolus-injected into the circuit. After 30 minutes, L-PAM was bolus-injected, and perfusion was continued for 1 hr. Temperatures were continuously monitored and adjusted to prevent the muscle temperature from increasing to >41.5°C.

At the end of perfusion, the circuit was washed out with .9% saline solution and refilled with Normosol-R solution (Fresenius-Kabi, Isola Della Scala, Italy). Cannulae were then removed, and the breaches on the vessels were repaired. Systemic protamine was given to reverse heparinization.

To minimize the risk of acute renal failure due to myoglobin precipitation, 18% mannitol solution, together with generous intravenous fluid support and urine alkalinization, was given during perfusion and the early postoperative course until myoglobin plasma levels returned to normal. Locoregional toxicity was graded according to the scale of Wieberdink et al. (Table 1),[15] and systemic toxicity was classified according to the World Health Organization system.

TABLE 1. *Locoregional toxicity evaluation scale for isolated limb perfusion*

Grade	Description
1	No subjective or objective evidence of reaction
2	Slight erythema or edema
3	Considerable erythema or edema with some blistering; slightly disturbed motility possible
4	Extensive epidermolysis or evident damage to the deep tissues, causing definite functional disturbances; threatening or manifest compartmental syndrome
5	Reaction that may necessitate amputation

Patient Follow-Up

Follow-up included physical examination at 1, 3, and 6 months after HILP and then every 6 months. Chest x-rays and hepatic and lymph node ultrasound scans were undertaken every 6 months. Total-body computed tomographic scan, brain magnetic resonance imaging, and positron emission tomography scan were used if there was a suspicion of distant metastases.

Tumor Response and Clinical Outcome Evaluation

The evaluation of tumor response was mainly clinical; the number of tumors and their greatest diameter were measured.[16] Soft tissue ultrasound scan with or without fine-needle aspiration was used to assess deep nodules suspected at physical examination; if nodules were too numerous, photographs of the affected limb were taken.

Tumor response was evaluated 3 months after HILP and was classified as follows: CR, disappearance of all clinical evidence of active tumor for a minimum of 4 weeks; partial response (PR), decrease of 50% or more in the sum of the product of the diameters of measured lesions for at least 1 month without any simultaneous increase in size or the appearance of any new lesions; no change, decrease in tumor size <50% or increase of tumor size <25%; and progressive disease, increase of >25% in the size of any measured lesion, appearance of a new tumor, or both. The clinical outcome of patients was assessed by analyzing local progression-free and overall survival, which was estimated with the Kaplan-Meier method.

RESULTS

From January 1997 to September 2002, 20 patients (14 women and 6 men; mean age, 63 years; range, 32–80 years) who met the previously reported eligibility criteria underwent lower-limb HILP with 1 mg of TNFα and 10 mg/L of L-PAM. Five patients were also treated with inguinoiliac lymphadenectomy for synchronous lymph node metastases. Eight (40%) of the 20 patients had already undergone L-PAM HILP and were then submitted to low-dose TNFα-based redo HILP for recurrence; the median interval between the 2 procedures was 12 months (range, 6–48 months).

Regarding HILP parameters, mean systemic leakage was .9% (range, 0%–3.9%); mean muscle and tumor temperature was 41.2°C (range, 41°C–41.5°C) and 41.4°C (range, 41.1°C–41.7°C), respectively. Patients who had disease recurrence or progression underwent surgical excision of nodules (n = 2), radiotherapy (n = 3), or systemic bio/chemotherapy (n = 13).

Postoperative Morbidity

There were no postoperative deaths, nor were there cases of significant systemic toxicity. During the early postoperative course, all patients had augmented plasmatic myoglobin levels, but none developed acute postoperative renal failure. The mean time to normal myoglobin values was 3 days (range, 2–8 days).

No compartmental syndromes were reported. One patient had acute limb ischemia from damage of the intima during cannulation; this required early reoperation and a femorofemoral polytetrafluoroethylene graft repair. Thirteen (65%) patients had grade 1 locoregional toxicity, 6 (30%) had grade 2, and 1 (5%) had grade 3. No patient required amputation because of toxicity.

Tumor Response and Clinical Outcome

Complete and partial tumor responses were observed in 14 (70%) and 5 (25%) cases. The overall response (CR plus PR) rate was therefore 95%. The remaining patient showed a tumor response of <50% (no change).

Overall and local progression–free survival curves are shown in Fig. 1. After a median follow-up of 18 months (range, 10–63 months), six (30%) patients are alive and disease free, four (20%) are alive with local disease recurrence, three (15%) are alive with local disease and distant metastases, and seven have died of distant metastases after developing locoregional recurrence (Table 2). To date, among patients who had a CR (n = 14), eight (57%) have developed local recurrence, and four of the five patients who had a PR have local disease progression. The median time to local progression was 10 months (range, 6–63 months); considering patients with

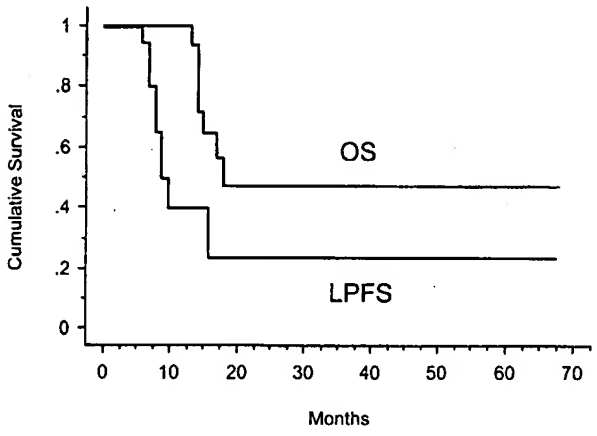

FIG. 1. Overall survival (OS) and local progression–free survival (LPFS) analysis of 20 patients who underwent hyperthermic isolated limb perfusion with low-dose tumor necrosis factor-α and melphalan for bulky in-transit melanoma metastases.

TABLE 2. *Tumor characteristics and clinical outcome in 20 patients who underwent hyperthermic isolated limb perfusion with low-dose TNFα and L-PAM for recurrent/ bulky in-transit melanoma metastases*

Patient (sex)	Tumor characteristics		Tumor response	Patient status
	No metastases	Maximum diameter (cm)		
1 (F)	25	1	CR	NED
2 (F)	20	.5	CR	NED
3 (F)	23	.5	PR	DOD[a]
4 (M)	19	.5	CR	NED
5 (F)	21	.5	CR	DOD[a]
6 (F)	17	1	CR	AWD
7 (F)	21	1.5	CR	DOD[a]
8 (M)	5	>3	CR	AWD
9 (F)	21	.5	PR	AWD[a]
10 (M)	17	1	CR	DOD[a]
11 (F)	20	.5	PR	DOD[a]
12 (M)	7	>3	PR	AWD[a]
13 (F)	19	1	CR	NED
14 (F)	4	>3	CR	DOD[a]
15 (F)	20	.5	NC	DOD[a]
16 (M)	18	.5	CR	AWD[a]
17 (F)	19	1	CR	NED
18 (F)	>30	.5	CR	AWD
19 (F)	19	.5	PR	AWD
20 (M)	21	.5	CR	NED

CR, complete response; NED, no evidence of disease; PR, partial response; DOD, dead of disease; AWD, alive with disease (local recurrence).

[a] Local and distant disease.

CR, the median local disease–free time was 16 months (range, 8–23 months).

DISCUSSION

The introduction of TNFα in HILP is considered an important breakthrough in the locoregional treatment of limb-confined solid tumors, and this cytokine has been approved by the European Drug Agency for the treatment of limb-threatening soft tissue sarcomas. Among the many uncontrolled trials in patients with melanoma that have been published, some report controversial results, mainly because of poor patient selection.[4] Fraker et al.[8] recently published the results of a randomized phase III study on 103 patients that showed an advantage in the arm treated with the association of L-PAM plus TNFα and IFNγ in terms of CR rate, whereas no significant improvement was observed in the overall response rate, the local recurrence rate, or overall survival. A tendency toward better responses in patients with bulky disease has also been observed, although not all the patients enrolled in this study had bulky disease. In this trial, as in most of the published series, TNFα was used at full dosage (4 and 3 mg for upper and lower limb, respectively) in combination with subcutaneous IFNγ.

In our series, the patient population was carefully selected in terms of tumor burden. Thus, we could analyze the activity of low-dose TNFα HILP in a quite homogeneous group of patients. The 70% CR rate reported by us is similar to that reported by Fraker et al., although we used much lower TNFα doses and hyperthermia. Furthermore, the median time to progression (10 months) in our series, which included only patients with bulky tumor, is comparable to that reported by other investigators who enrolled patients regardless of tumor burden.[8,16] Finally, locoregional toxicity was quite limited: no patient required amputation or had permanent toxicity, and most patients (95%) had mild (grade 1 or 2) locoregional toxicity. The high activity of this novel therapeutic regimen may depend on some pharmacological and biological factors. The high response rate observed in this series might be related to the fact that 1 mg of TNFα is sufficient to reach in vivo the saturation of TNFα receptors, a phenomenon already described in vitro.[17] Furthermore, on performing a pharmacokinetic study with low-dose TNFα HILP for advanced limb sarcomas (unpublished data), we reported TNFα perfusate concentrations 20-fold greater than those considered cytotoxic in vitro.[18] These levels remained steady during HILP, supporting the hypothesis that 1 mg of cytokine is enough to saturate the entire uptake capacity of the limb. However, it is well known that heat, cytostatics, and TNFα act synergistically and that TNFα itself increases L-PAM penetration into tumor.[3,19–21] Finally, because tumor vasculature is the main target of TNFα, bulky tumors may be more sensitive to drug regimens containing this cytokine because of their high density of neovascularization.

Although TNFα seems to play a key role in the management of patients with in-transit melanoma metastases, two major drawbacks must be kept in mind: high costs and potentially lethal systemic toxicity. Regarding costs, TNFα is available on the market at a price of €2300/mg, with a cost of approximately €6900 and €9200 at full dosage (3–4 mg). Thus, our HILP drug regimen (1 mg of TNFα) contributes to cutting costs.

Regarding systemic toxicity, the mainstay is accurate leakage control and monitoring. Of course, the lower the TNFα dose, the lower the risk of severe toxicity.

Overall, these considerations support the use of TNFα at lower doses than those commonly used. However, a larger comparative study is warranted to confirm the good results we obtained in this subset of patients affected with locally advanced melanoma.

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